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Structural and stereochemical diversity in prenylated indole alkaloids containing the bicyclo [2.2.2]diazaoctane ring system from marine and terrestrial fungi

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Abstract

Various fungi of the genera *Aspergillus*, *Penicillium*, and *Malbranchea* produce prenylated indole alkaloids possessing a bicyclo[2.2.2]diazaoctane ring system. After the discovery of distinct enantiomers of the natural alkaloids stephacidin A and notoamide B, from *A. protuberus* MF297-2 and *A. amoenus* NRRL 35660, another fungi, *A. taichungensis*, was found to produce their diastereomers, 6-*epi*-stephacidin A and versicolamide B, as major metabolites. Distinct enantiomers of stephacidin A and 6-*epi*-stephacidin A may be derived from a common precursor, notoamide S, by enzymes that form a bicyclo[2.2.2] diazaoctane core *via* a putative intramolecular hetero-Diels–Alder cycloaddition. This review provides our current understanding of the structural and stereochemical homologies and disparities of these alkaloids. Through the deployment of biomimetic syntheses, whole-genome sequencing, and biochemical studies, a unified biogenesis of both the dioxopiperazine and the monooxopiperazine families of prenylated indole alkaloids constituted of bicyclo[2.2.2]diazaoctane ring systems is presented.

1. Introduction

The isolation of natural products from marine and terrestrial-derived fungal species of the *Aspergillus*, *Penicillium* and related genera has allowed for a widely studied diverse class of

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Conflicts of interest

There are no conflicts to declare.

secondary metabolites. In particular, various prenylated indole alkaloids have been discovered, including the brevianamides,^{1–7} marcfortines,^{8,9} paraherquamides,^{10–14} penicimutamides,¹⁵ sclerotiamide,¹⁶ asperparalines,¹⁷ avrainvillamide,^{18,19} stephacidins,²⁰ malbrancheamides,^{21–23} notoamides,^{24–27} versicolamide B,²⁸ chrysogenamide,²⁹ and recently the taichunamides (Fig. 1).³⁰ The presence of a bicyclo[2.2.2]diazaoctane ring system and densely functionalized indole-derived units form the bases for the structural allure of these naturally occurring metabolites. Many of these alkaloids have been found to show a variety of biological activity including insecticidal, cytotoxic, anthelmintic, calmodulin inhibition,³¹ osteoclast inhibition, and antibacterial properties.^{1–3,5,8–30,32–34} Reviews in this area have included the studies on the discovery of unexpected enantiomeric and diastereomeric relationships (Fig. 2) (see Section 2) as well as developments in the biosynthesis of these compounds (see Section 3), and biomimetic total syntheses (see Section 4).^{35–41} Herein, this review will focus on the biogenesis of the notoamides and their congeners, and provide the current understanding of the structural and stereochemical homologies and disparities that are particularly intriguing from a biosynthetic perspective.

Elucidation of the biogenesis of these compounds became of particular interest upon the isolation of antipodal metabolites from different species of the *Aspergillus* genus from both marine and terrestrial sources. Initial studies from the Williams laboratory⁴² were based on seminal work first reported by Sammes⁴³ and Birch,⁴⁴ and demonstrated that these natural metabolites arise from L-tryptophan, one or two isoprene units, and L-proline, β-methylproline, or pipecolic acid.

The first proposal concerning the biogenesis of the bicyclo [2.2.2]diazaoctane ring system was that of Sammes in 1970,⁴³ wherein the intermediacy of an intramolecular hetero Diels–Alder construction through the agency of an azadiene species was proposed (left, Fig. 3). Shortly thereafter, Birch inferred in 1971 (ref. 45) the intermediacy of an epidithiapiperazinedione species, with net extrusion of S₂ to form this ring system (right, Fig. 3), although the nature of the C–C bond formations as being heterolytic, radical, or stepwise were not further discussed.

At the time of the Sammes and Birch publications in the early 1970's, the absolute configuration of brevianamide B had not been rigorously assigned, but was thought to be of the same absolute sense as that of brevianamide A and being diastereomeric at the spiro-indoxyl stereogenic center. The Williams laboratory, in 1988,⁴⁶ completed the first synthesis of (–)-brevianamide B and re-assigned the absolute configuration of natural (+)-brevianamide B, as depicted in Fig. 1.^{47,48} This revealed that brevianamides A and B have a pseudo-enantiomeric relationship (that is, the tricyclic portion constituted with the bicyclo[2.2.2]diazaoctane ring system are enantiomeric, but brevianamides A and B are technically diastereomers) which immediately raised provocative questions regarding the biogenesis of these natural substances in *Penicillium brevicompactum*. Williams suggested a modification to the intramolecular hetero Diels–Alder proposal, originally postulated by Sammes,⁴³ as shown in Scheme 1.^{39,49,50} The differences between the Sammes and Williams biogenetic constructions involves the timing of the indole oxidation relative to the IMDA reaction. The Sammes proposal invokes a spiro-5 IMDA construction whereas the Williams alternative requires a spiro-6 IMDA. The Williams lab interrogated their alternative

hypothesis through the synthesis and attempted precursor incorporation of DL-1. This potential metabolite was not incorporated into either brevianamide A nor brevianamide B in *Penicillium brevicompactum*,^{51,52} leaving the Sammes proposal as the most plausible. However, advances in confirming the assembly details of these fungal indole alkaloids were only made possible with identification of the notoamide gene cluster (see Section 3) as well as through labeled precursor incorporation studies of anticipated intermediates (see Section 2).

A deeper understanding of the enantio- and diastereo-divergence of the biosynthetic pathways exhibited by these closely related fungi, has led to the proposal of the functional presence of enzymes putatively mediating a Diels–Alder-type construction, an enzymatic function that is now receiving considerable attention from a number of laboratories investigating natural products biosynthetic pathways.⁵³ Theoretical^{54,55} and experimental^{56–61} studies from our laboratories have provided corroborating evidence that an enzyme-mediated IMDA reaction is operative in the biosynthesis of the bicyclo [2.2.2]diazaoctane ring systems.^{38,53}

2. Fungal metabolites and biosynthetic studies of prenylated bicyclo[2.2.2]diazaoctane indole alkaloids

As to be discussed in detail below, there exists striking stereochemical similarities and differences between various members of this family of alkaloids for which biogenetic constructions need to accommodate. With respect to the construction of the bicyclo[2.2.2]diazaoctane ring system, the progenitor dioxopiperazine **4** (X = O) (or, for the paraherquamides, marcfortines, asperparalines, and malbrancheamides, the progenitor monooxopiperazine, **4**, X = H₂) suffers a net two-electron oxidation allowing the pendant isoprene-derived vinyl moiety to form the two new C–C bonds of the bicyclo[2.2.2]diazaoctane ring system. Four distinct diastereomeric transition states for this process can be operative resulting in the *syn*- (**5**) or *anti*- (**6**) diastereomeric relationships and a more subtle outcome being the manifestation of top-face or bottom-face cycloaddition as generically summarized in Fig. 4.

These stereochemical outcomes can be specifically identified in the known metabolic fates of notoamide S as illustrated in Scheme 2.^{61,63} Various metabolites have been identified, which were biosynthesized through both enantiomers of notoamide T and 6-*epi*-notoamide T.^{27,30,61,63–65} It has been suggested that all four of these substances arise *via* the intermediacy of common achiral azadiene species **7** (Scheme 2).

2.1 Isolation of prenylated indole alkaloids from the marine- derived fungus *A. protuberus* MF297-2 and the first proposal of the biogenesis of these alkaloids

The marine-derived fungus *A. protuberus* MF297-2 was isolated from the mussel *Mytilus edulis galloprovincialis*, which was collected from the Noto peninsula (Japan) by the Tsukamoto laboratory. The fungal culture showed the activity of cell cycle inhibition in HeLa cells and the bioassay-guided purification afforded a fungal toxin, sterigmatocystin, as a cell cycle inhibitor along with seven prenylated indole alkaloids. Structure elucidation

revealed that four of these were new alkaloids, notoamides A–D, and three were known alkaloids, sclerotiamide, stephacidin A, and deoxybrevianamide E.^{1,24} The structural relationship of these alkaloids led to the first proposal of the biosynthetic pathway illustrated in Scheme 3.^{66,67} Notoamide E was suggested to be a key precursor for this family, although it had not yet been isolated from the culture. The compounds containing a bicyclo [2.2.2]diazaoctane nucleus, namely stephacidin A, notoamides A and B, and sclerotiamide, were hypothesized to be produced from notoamide E through an achiral azadiene intermediate **8** by the IMDA reaction.

2.2 Precursor incorporation studies of notoamide E and structurally novel metabolites from *A. protuberus* MF297-2

Since notoamide E was identified as a key intermediate in the biosynthesis of prenylated indole alkaloids in *A. protuberus*, Tsukamoto, *et al.* searched for notoamide E in the culture and found that it existed only in the fifth day of culture growth and immediately disappeared the next day.^{68,69} These results clearly indicated that notoamide E was produced by the fungus in the early phase of growth and was presumably rapidly converted to other downstream metabolites. Subsequently, precursor incorporation experiments with synthetic, [¹³C]₂-notoamide E were carried out.^{68,71} Surprisingly, it was converted only to notoamides C and D as well as four new alkaloids, 3-*epi*-notoamides C and notoamides E2–E4, but not to the alkaloids containing a bicyclo[2.2.2]diazaoctane core, such as stephacidin A, notoamides A and B, nor sclerotiamide (Fig. 5).

Furthermore, in a precursor incorporation experiment, [¹³C]₂-(+)-stephacidin A was, as expected, converted to (–)-notoamide B and (–)-sclerotiamide (Scheme 3).⁷⁰ The four new alkaloids (3-*epi*-notoamide C and notoamides E2–E4) were not detected as metabolites from this organism in the normal nutrient-rich medium. Among these, the structure of notoamide E4 is particularly interesting, and two possible biosynthetic pathways from notoamide E can reasonably accommodate the biogenesis of these species (Scheme 4).⁶⁸ One of the possible pathways would proceed *via* dioxetane intermediate **9**, followed by fragmentation to the corresponding kynurenine derivative **10**. Then, cyclization of **10** would afford the eight-membered ring derivative, notoamide E4. An alternative pathway is *via* peroxide species **11**, which would be produced by proton-assisted nucleophilic attack of the indole 3-position on molecular oxygen. The results of this precursor incorporation experiment reveals that the addition of excess [¹³C]₂-notoamide E (an endogenous metabolite) above the concentration levels of this metabolite normally produced, appears to alter the secondary metabolite profile of this organism. This provocative result warrants further investigation into how notoamide E, at elevated concentrations, must trigger the expression of dormant tailoring genes present that are not expressed under normal growth conditions.

Tsukamoto, *et al.* succeeded in the isolation of notoamides M²⁶ and Q,²⁷ which have hydroxy and methoxyl groups at C-17 positions, respectively (Scheme 5). These metabolites represent the oxidation state of the putative azadiene species that have been strongly implicated in the construction of the bicyclo [2.2.2]diazaoctane ring system. Thus, it is possible that formation of azadiene intermediate (D), proceeds through oxidation at C-17 of dioxopiperazine A to B, followed by loss of water or methanol and tautomerization to D

(Scheme 5). Alternatively, notoamides M and Q, might be artifacts of hydration/capture of azadiene species (C) and experiments to interrogate this possibility are being investigated.

Tsukamoto, *et al.* isolated eighteen new prenylated indole alkaloids, notoamides A–R,^{24–27} from the marine fungus *A. protuberus*. Among these, carbon frameworks of notoamides L²⁶ and O²⁷ were novel in the family of prenylated indole alkaloids and notoamides N²⁶ and P²⁷ contained chlorine and bromine atoms, respectively (Fig. 6). Notoamide L is the first metabolite containing twenty-five carbons in this family, while all other alkaloids possess twenty-six carbons. In the structure of notoamide L, the C-2 carbon derived from tryptophan has been removed.

Notoamide O possesses a novel hemiacetal/hemiaminal ether functionality hitherto unknown among this family. Possibly, notoamides L and O may be derived from (+)-stephacidin A (Scheme 6). In the biogenesis of notoamide L, singlet oxygen reaction at the indole 2,3-position of (+)-stephacidin A would afford a dioxetane intermediate **12** followed by fragmentation to kynurenine **13**. After hydrolysis of the amide bond of **13**, oxidation at C-24 followed by decarboxylative dehydration would afford notoamide L.²⁶ On the other hand, stephacidin A may be converted to aspergamide B⁷² via notoamide R. Oxidative cleavage of the tri-substituted alkene in aspergamide B would generate aldehyde hydrate **16**; simple ring closure would afford notoamide O.²⁷ Notoamide N is one of the rare chlorinated members of the family of prenylated indole alkaloids and this is the third chlorinated derivative following malbrancheamide,²¹ malbrancheamide B²² and spiromalbramide.²³ On the other hand notoamide P is the first brominated member of this family.^{27,65} The existence of a halogenase system in these fungi is of interest and the identification of the genes responsible for the halogenations merits further investigation.

2.3 Isolation of enantiomers of stephacidin A and notoamide B from the terrestrial fungus *Aspergillus amoenus* NRRL 35660

The Gloer laboratory originally isolated (–)-stephacidin A and (+)-notoamide B from the terrestrial fungus *A. amoenus* (formerly *A. versicolor*) NRRL 35660, which was isolated from a basidioma of *Ganoderma australe* collected in a Hawaiian forest, along with a new alkaloid, (+)-versicolamide B (Fig. 2).²⁸ (+)-Stephacidin A was originally isolated from the terrestrial fungus *A. ochraceus* by Bristol-Myers Squibb²⁰ and subsequently, (+)-stephacidin A and (–)-notoamide B were isolated from the marine-derived fungus *A. protuberus* MF297-2, by the Tsukamoto laboratory.²⁴ These facts reveal that these closely related *Aspergillus* fungi biosynthesize the opposite enantiomers of stephacidin A and notoamide B and it has been confirmed that the optical purity of these metabolites are >99%. The initial hypothesis for rationalizing how these opposite enantiomers might arise, is illustrated in Scheme 7 and invokes the oxidation of notoamide E, to the achiral azadiene species **8**, followed by an entirely enantio-selective cycloaddition from this common precursor.²⁸ Although [¹³C]₂–stephacidin A was converted to [¹³C]₂–notoamide B,⁷⁰ as discussed above, the incorporation of [¹³C]₂–notoamide E into stephacidin A and notoamide B, however, was not observed in *A. amoenus*⁷³ nor *A. protuberus*.⁶⁸ These results suggested alternate timing of the pyran and bicyclo[2.2.2]diazaoctane ring constructions as discussed below.

2.4 An alternative enantiodivergent biosynthetic pathway for stephacidin A biogenesis

Since [^{13}C]₂-notoamide E was not incorporated into stephacidin A in either *A. protuberus* MF297-2 nor *A. amoenus* NRRL 35660, other testable hypotheses needed to be devised. The pathway from deoxybrevianamide E to stephacidin A contains four steps: the putative IMDA, an oxidation at C-6, prenylation, and final oxidation/cyclization to form a pyran ring (Scheme 8). From the results of the precursor incorporation experiments with notoamide E, three compounds, namely deoxybrevianamide E, 6-hydroxy-deoxybrevianamide E, and notoamide S, appeared to be potential precursors for IMDA reactions, although notoamide S had not yet been isolated from any species of the *Aspergillus* fungi.⁷⁴ The incorporation experiments of synthetic [^{13}C]₂-[^{15}N]-deoxybrevianamide E⁷⁵ and [^{13}C]₂-[^{15}N]-6-hydroxydeoxybrevianamide E^{75,76} afforded notoamides C and D but not stephacidin A nor notoamides A and B. Next, the administration of synthetic [^{13}C]₂-[^{15}N]₂-notoamide S in *A. amoenus* was examined and it was found to be incorporated into (–)-stephacidin A (6.2% incorporation), (+)-notoamide B (6.4% incorporation), and (+)-versicolamide B (6.5% incorporation), together with notoamides C (6.4% incorporation) and D (6.2% incorporation) (Fig. 7).⁷⁷ These results clearly indicated that the construction of the bicyclo[2.2.2]diazaoctane ring system occurs prior to the formation of the pyran ring. Although notoamides S and T had not previously been found in the culture extract of *A. amoenus*, Kato, *et al.*, have recently identified notoamide S as a minor metabolite of *A. amoenus*.⁶¹

2.5 The incorporation of notoamide T and 6-*epi*-notoamide T

Since notoamide S was observed to be converted to compounds containing a bicyclo[2.2.2]diazaoctane core *via* notoamide T and 6-*epi*-notoamide T (Scheme 2), the precursor incorporation experiments of these two alkaloids were performed. Unexpectedly, the administration of synthetic [^{13}C]₂-(±)-notoamide T in *A. protuberus* afforded racemic mixtures of stephacidin A, notoamide B, notoamide F, notoamide R, and a new compound, notoamide T2 (Fig. 8A).⁶³ On the other hand, synthetic [^{13}C]₂-6-*epi*-(±)-notoamide T was converted to racemic mixtures of versicolamide B and eight new compounds, 6-*epi*-notoamides T3–T8 and 6-*epi*-notoamide I, in a trace element solution (Fig. 8B) and afforded racemic mixtures of 6-*epi*-stephacidin A and four new compounds, 6-*epi*-notoamides T9–T12, on trace element agar plates (Fig. 8C).⁶⁴

Among them, notoamide T2, 6-*epi*-notoamides T3–T12, and 6-*epi*-notoamide I were not produced by the fungus under normal culture conditions to any detectable extent.^{63,64} These data suggest that the (presumably) endogenous, natural metabolites [^{13}C]₂-(±)-notoamide T and [^{13}C]₂-(±)-6-*epi*-notoamide T activated the expression of dormant tailoring genes that expands the metabolome of the producing organism. While notoamide T and 6-*epi*-notoamide T are presumed to be natural, endogenous metabolites in this organism, the precursor incorporation experiment with the easily accessible synthetic, racemic labeled substances was performed. In order to further interrogate this fascinating observation, sufficient quantities of the respective optically pure enantiomers of each will be required to query whether the metabolite profile alteration is triggered by the presence of the unnatural enantiomer for this producing organism.

Of particular intrigue, 6-*epi*-notoamides T3 and T4 contain unprecedented structures wherein the aromatic benzenoid ring of the tryptophan moiety has suffered oxidative amination, culminating in the formation of the new fused oxazole and 2-oxazolone moieties, respectively. One possible mechanism for the oxidative amination of the tryptophan moiety is suggested in Scheme 9. Oxidation and subsequent pinacol rearrangement of [^{13}C] $_2$ -(\pm)-6-*epi*-notoamide T would form the spiro-oxindole, [^{13}C] $_2$ -(\pm)-6-*epi*-notoamide T9. Successive oxidation and incorporation of glycine into **20** to form imine **21**, followed by decarboxylation, provides **22** that must cyclize (to **23**) and then be oxidized to the oxazoline moiety of T3. Oxidation of the oxazoline of T3 would produce oxazolidinone in 6-*epi*-notoamide T4.⁶⁴ It is reasonable to assume that the initial oxidation of the 2,3-disubstituted indole of 6-*epi*-notoamide T is conducted by NotI that Li, *et al.*, previously predicted this oxidation and spiro-rearrangement.⁶² The downstream transformations from 6-*epi*-notoamide T9, requires a minimum of six enzyme-catalyzed reactions: (1) aromatic hydroxylase of T9 to **19**; (2) a catechol oxidase to **20**; (3) glycine aminotransferase to **21**; (4) decarboxylase/cyclase to **23**; (5) oxazolidine oxidase to T3; and (6) oxazolidinone oxidase to T4. The complex array of transformations required to construct T3 and T4 from 6-*epi*-notoamide T mandates that multiple dormant tailoring genes extant in this organism were activated by the presence of one (or both) enantiomers of 6-*epi*-notoamide T. Significant effort will need to be devoted to elucidating the nature of these remarkable transformations that have significant implications for the plasticity of secondary metabolomes in Nature.

2.6 Isolation of an enantiomeric mixture of 6-*epi*-stephacidin A from *A. amoenus* NRRL 35660

The metabolic profiles of *A. protuberus* MF297-2 and *A. amoenus*. NRRL 35660 produced the opposite enantiomers of stephacidin A and notoamide B along with the same enantiomer of (+)-versicolamide B. (–)-Versicolamide B has yet to be detected as a natural metabolite in any of the fungi that have been examined to date and poses an enigma (Fig. 2). (+)-6-*epi*-Stephacidin A is the likely precursor of (+)-versicolamide B and was obtained from the culture of *A. protuberus* (Scheme 10).⁶⁴ However, 6-*epi*-stephacidin A, which was isolated from *A. amoenus*, was found to be an enantiomeric mixture enriched with the (–)-isomer (1 : 2.4).⁶¹ This result clearly suggested that, in *A. amoenus*, notoamide S was converted to both (+)- and (–)-6-*epi*-stephacidin A and subsequently, only the (+)-isomer was converted into (+)-versicolamide B (Scheme 10). This result mandates that *A. amoenus* contains a highly enantio-specific indole oxidase that exclusively transforms the (+)-enantiomer of 6-*epi*-stephacidin A to (+)-versicolamide B, but apparently does not bind or oxidize the (–)-enantiomer of 6-*epi*-stephacidin A. While the formation of an enantiomerically-enriched metabolite has not yet been observed in any of the other fungal species examined, this begs the question of why this occurs in *A. amoenus*. The lack of optical purity of 6-*epi*-stephacidin A obtained in *A. amoenus* also requires an explanation and it is to such questions, that our labs have recently started investigations.

2.7 Isolation of taichunamides A–G from *Aspergillus taichungensis* IBT 19404: biogenetic considerations

The majority of the prenylated indole alkaloids thus far identified containing the bicyclo[2.2.2]diazaoctane ring system possess the *syn*-configuration (the *syn*- and *anti*-

relationship is based on the relative configuration of H21 with respect to the bridging secondary amide; see Fig. 4 and 9). Distinct enantiomers of notoamide B and stephacidin A are the main metabolites present in *A. protuberus* and *A. amoenus*, and (+)-versicolamide B, a minor metabolite, was the first alkaloid identified within this family with the corresponding *anti*-configuration. In 2013, Cai *et al.* isolated (+)-versicolamide B and (+)-6-*epi*-stephacidin A as major metabolites from *A. taichungensis* ZHN-7-07.⁷⁸ Kagiya, *et al.*, have also investigated the isolation of alkaloids from *A. taichungensis* IBT 19404 collected from the soil in Taiwan and succeeded in isolating seven indole alkaloids with unprecedented structures and have named these substances taichunamides A–G (Fig. 10); all of these natural alkaloids possess the *anti*-bicyclo[2.2.2]diazaoctane core.³⁰ Although the structure of taichunamide A was initially assigned to contain a very rare azetidine unit, recently it was revised as a 3-hydroxyindolenine.⁷⁹ Taichunamide B was found to be constituted with a very rare 4-pyridone unit. In DMSO-*d*₆ taichunamide B appears to be an equilibrium mixture of two tautomeric entities: a 4-pyridone and 4-pyridol, in a ratio of 3 : 1. This tautomeric equilibrium is highly solvent-dependent where it was observed a single keto or enol form was observed in CD₃OD or acetone-*d*₆, respectively. Taichunamides C and D contain endoperoxide and methylsulfonyl units, respectively.

These new alkaloids, especially taichunamide B, constitute hitherto unknown structural arrays derived from tailoring of the tryptophan moiety and therefore must deploy a series of fascinating biochemical bond constructions. Biogenetic implications for taichunamides B and E as well as (+)-versicolamide B are represented in Scheme 11.³⁰ β -Face oxidation (*anti*- to the bridged amide) of 6-*epi*-stephacidin A allows for the formation of a non-isolatable epoxide intermediate **24** which subsequently undergoes a pinacol rearrangement within the active site to form the spirooxindole in (+)-versicolamide B. Oxidation at the α -face (*syn* to the bridged amide) followed by pinacol rearrangement would allow for formation of taichunamide E. Compared with β -face oxidation, α -face oxidation is more sterically demanding with N19, as reflected by the metabolite ratios of (+)-versicolamide B (15.9 mg)/(+)-versicolamide C^{30,80} (a N1-OH derivative of versicolamide B)⁸¹ (240 mg) *versus* taichunamide E (0.43 mg). Although, in the biomimetic synthesis that deployed the IMDA reaction, taichunamide E was identified along with (+)-versicolamide B in a ratio of 1 : 1.4; this is the first report of its isolation from the fungal culture.³⁰ Also, formation of peroxide **26** by oxidation of (+)-6-*epi*-stephacidin A would ultimately lead to the ring system found in taichunamide B, theoretically through compound **27**.

A wide structural array of prenylated indole alkaloids have been isolated from the genera of *Aspergillus* and *Penicillium* to date, yet their respective carbon frameworks are very unique. The isolation of these compounds from *A. taichungensis* has greatly expanded the library of these secondary metabolites. Future biochemical studies and genome mining will aid in the identification of genes or gene clusters responsible for the evolution of these unprecedented metabolites.

2.8 Biogenetic implications of prenylated indole alkaloids in the *Aspergillus* genus

To date, numerous fungal species in the *Aspergillus* genus, including *A. ochraceus*, *A. protuberus* MF297-2, *A. amoenus* NRRL 35660, and *A. taichungensis* IBT 19404, described

in this article that produce prenylated indole alkaloids, bearing the dioxopiperazine-based bicyclo[2.2.2]diazaoctane core most likely biosynthesized by the oxidative cycloaddition reaction of the common precursor, notoamide S (Scheme 12), have been identified. Each organism displays its own unique stereochemical signature wherein the absolute and relative stereochemistry within these molecular frameworks have been constructed are evident. In *A. protuberus*, the orientation of the dienophile to the diene in pathways **a** (main) and **b** (minor), respectively, leads to the construction of the corresponding *exo*- and *endo*-products namely, (+)-stephacidin A/(–)-notoamide B and (+)-6-*epi*-stephacidin A/(+)-versicolamide B, respectively. On the other hand, in *A. taichungensis*, the construction of the bicyclo[2.2.2]diazaoctane ring system favors the formation of the C-6-*epi*-diastereomeric *via* pathway **b** (main) and **c** (minor).³⁰ Efforts to clarify the genetic and biochemical bases and relationships between the respective biosynthetic gene clusters are currently under intense investigation in our laboratories.

3. Genomic insights

In order to obtain a deeper understanding of the biogeneses of these structurally fascinating natural alkaloids, and in particular, to obtain a higher level elucidation of the enantioselectivity and diastereoselectivity evident in the biogenesis of the stephacidins and notoamides, the genomes of ten fungi known to produce these alkaloids have been sequenced and the identification of relevant biosynthetic gene clusters (Fig. 11) has been realized. The mining of *ftmA*, a NRPS gene from the fumitremorgin *Aspergillus* species, *A. fumigatus*, and production of brevianamide F by heterologous gene expression in 2006 by Turner *et al.*,⁸² allowed Ding, *et al.*, to identify the notoamide gene cluster in *A. protuberus*.⁸³ Due to the crucial role brevianamide F plays in the early biogenesis of an enormous range of prenylated indole alkaloids within the *Aspergillus* genus, it was originally assumed that *ftmA*, or a comparable homolog, would also participate in biosynthetic transformations of *A. protuberus*. Using Roche 454FLX technology and *ftmA* to probe for homologous genes, *notE* was identified as the NRPS module analogous to *ftmA*, sharing a 47% nucleotide identity, in the *A. protuberus* species.⁸³ Fig. 12 represents the (+)- and (–)-notoamide-producing gene clusters identified by genome sequencing and bioinformatics.⁸³

Bioinformatics analysis⁸³ and deep annotation, has revealed a number of important facts and relationships between *A. protuberus* and *A. amoenus* that produce the opposite enantiomers of stephacidin A and notoamide B. First, it is clear from the structure and directionality of the open-reading frames of genes within the gene clusters, that the gene clusters and thus the organisms are orthologous (ancestrally related through a speciation event that occurred at some time during their evolutionary history). The high level of nucleotide identity overall between the gene clusters is almost 71% and within individual genes, the homology with respect to amino acid identity or similarity, is as high as 95% (for example NotC). Functions have been assigned to a number of genes within these orthologous gene clusters and are identified in Fig. 12, Scheme 13, and Table 1 which reflects our current understanding and hypotheses regarding the biogenesis of the enantiomeric pairs.^{63,83}

Li, *et al.*, have successfully been able to clone and functionally express the following enzymes: NotF, NotG, NotC, NotD, NotD', NotI and NotI'.^{62,83} Table 1 provides a

summary of the relevant data on each individual gene and the heterologous host in which the protein was functionally expressed.

Li, *et al.*, have tentatively assigned roles to other genes in the notoamide gene clusters as follows. NotG appears to be a cytochrome P450 oxidase responsible for the transformation of deoxybrevianamide E into 6-hydroxydeoxybrevianamide E, which was deduced through a process of elimination by comparative analysis with several fungal gene clusters.^{62,83} The other cytochrome P450, NotH is the current most plausible candidate for the oxidative transformation of notoamide S into notoamide T and 6-*epi*-notoamide T.⁸³ Much to our chagrin, NotH and NotH', which is currently the focus of where the key enantiodivergence occurs, is a membrane-associated cytochrome P450 that has proven recalcitrant to functional expression in a heterologous host and constitutes the focus of our current efforts.

Biochemical characterization, along with isotopically labeled precursor incorporation studies (*vide supra*), has determined the roles of NotF and NotC as aromatic prenyltransferases.⁸³ Michaelis–Menten kinetics and isotopically labeled precursor incorporation studies have successfully demonstrated the substrate selectivity of the NotF and NotC enzymes. The reverse-prenylation of brevianamide F into deoxybrevianamide E, the first committed step of the post-NRPS tailoring cascade, is catalyzed by NotF. The subsequent normal prenylation of 6-hydroxydeoxybrevianamide E into notoamide S is catalyzed specifically by NotC.⁸³

NotB and NotI are flavin-dependent monooxygenases (FMO) Biochemical characterization of NotB has determined its role as an FAD-dependent oxidase for converting notoamide E into notoamides C and D (Scheme 14).⁷⁷ Curiously, 3-*epi*-notoamide C is not observed as a product when notoamide E is exposed to recombinant NotB. 3-*epi*-Notoamide C has been previously isolated from a precursor incorporation experiment with notoamide E as an added substrate, but has not been directly isolated from fermentation extracts of *Aspergillus protuberus*.⁶⁸ NotI and NotI' which are also flavin-dependent oxygenases, are predicted to catalyze the oxidative spiro-transformation of stephacidin A into notoamide B.⁸³ The details of how NotI and NotI' discriminate between the respective enantiomers of stephacidin A and the conversion into the corresponding enantiomers of notoamide B is of great interest, but has not yet been reported.

4. Biomimetic total syntheses

With the objective of interrogating the validity of the proposed biosynthetic intramolecular hetero Diels–Alder construction of the characteristic bicyclo[2.2.2]diazaoctane ring nucleus, Williams, *et al.*, have investigated the laboratory version of the IMDA construction which successfully enabled biomimetic syntheses of numerous members of these natural alkaloids. These investigations provided additional insights as to the intrinsic facial bias of this cycloaddition with respect to the *syn*–*anti*-diastereoselectivity.^{56–60} Theoretical calculations on these azadiene IMDA reactions published by our collaborator Domingo *et al.*,^{54,55} provide an important backdrop to the laboratory and putative biosynthetic constructions. The strategies for generating the key azadiene species evolved to ever more efficient protocols over the years and are shown here chronologically. Our first successful biomimetic synthesis was that of *D,L*-brevianamide B, as shown in Scheme 15, where DDQ oxidation of lactam

ether **31** was employed to provide the unsaturated proline derivative **32**.⁸⁵ Base-mediated tautomerization generated the desired azadiene species, that proved stable enough to observe by ¹H NMR, but spontaneously suffered IMDA cycloaddition to provide an approximately 2 : 1 *syn* : *anti* mixture of cycloadducts **34** and **35**, respectively. Peracid oxidation to the corresponding diastereochemically pure 3-hydroxyindolenines (**36** and **37**) followed by base-mediated pinacol-type spiro-rearrangement and final acidic removal of the lactim ether furnished D,L-brevianamide B from **37** and the corresponding *syn*-diastereomer **38**.⁸⁵

A second-generation synthesis, detailed in Scheme 16, generated the azadiene directly from an α -ketoamide proline amide species (*i.e.*, **42**).⁸⁶ The IMDA reaction, carried out under strongly Lewis acidic conditions, interestingly provided the desired *anti*-cycloadduct **44**, exclusively. Fisher indole synthesis to **46** and final peracid oxidation to the indoxyl provided D,L-brevianamide B.⁸⁶

The most efficient biomimetic synthesis of brevianamide B that has been developed and reported to date, relied on using β -hydroxyproline as a surrogate representing the oxidation state of the azadiene, as shown in Scheme 17.^{60,85} Reverse-prenylated tryptophan species **47** was coupled with β -hydroxyproline ethyl ester to give dipeptide **48** that was deprotected and cyclized to **49**. Mitsunobu dehydration of **49** in dichloromethane at 40 °C led directly to the production of meta-stable azadiene species **50** that spontaneously underwent IMDA cycloaddition to give a 2.1 : 1, *syn* : *anti* diastereomeric mixture that were readily separated by chromatography. The minor *anti*-cycloadduct **46** was subjected to peracid oxidation as above and rearranged under basic conditions furnishing D,L-brevianamide B. It is important to note that the intrinsic facial bias of the IMDA cycloadditions in all cases, was about 2 : 1 favoring the *syn*-diastereomer and in good agreement with theoretical predictions published by our collaborator Prof. Domingo (see below). The intrinsic facial bias of these IMDA reactions, have been interrogated by Domingo, *et al.*, quantum chemically at the B3LYP/6-31G* level.⁵⁴ He has examined both the dioxopiperazine-based azadienes (**51b**; **54b**) relevant to the stephacidin and notoamide families, as well as the corresponding monooxopiperazine-based azadienes (**51a**; **54a**) relevant to the paraherquamide, malbrancheamide and related family members.

As depicted in Scheme 18, two main substrate platforms were evaluated: the spiro-5 mode, wherein the indole has been oxidized to the corresponding oxindole species and the *spiro*-6 mode, wherein the indole has yet to suffer oxidative tailoring.⁵⁴ Strikingly, it was found that the *spiro*-5 mode greatly favors the *anti*-diastereomer (**52**) over the corresponding *syn*-diastereomer (**53**) by 4–7 kcal mol⁻¹. In contradistinction, the corresponding *spiro*-6 mode from **54**, revealed a modest preference for the *syn*- diastereomers **56** by about 1 kcal mol⁻¹.⁵⁴ These theoretical predictions have tracked quite well with experiment as can be seen from the modest *syn* : *anti* diastereoselectivities for the laboratory *spiro*-6 systems described above and below, where the *syn* : *anti* ratio is typically around 2.5 : 1. On the other hand, the experimentally observed *spiro*-5 cycloadditions have to date, yielded exclusively the *anti*-diastereomers, which will be detailed below.

The biomimetic syntheses developed above for brevianamide B, set the stage for deploying this technology to prepare more complex members of this family. The Williams lab has been

able to successfully devise a biomimetic total synthesis of stephacidin A and its C-6-epimer, and more recently demonstrated C6-*epi*-stephacidin A to be a natural metabolite and, as noted above, these stephacidin A congeners are the direct biosynthetic precursors to notoamide B and versicolamide B, respectively. Subsequent to the biomimetic stephacidin A synthesis, these workers have been able to readily adopt this approach for successful biomimetic total syntheses of premalbrancheamide,⁸⁷ malbrancheamide,⁸⁸ spiromalbrancheamide,⁸⁹ marcfortine C,⁹⁰ notoamide T and its C-6-epimer,⁶³ also now known to be a natural metabolite as described above.

As shown in Scheme 19, the reverse-prenylated tryptophan derivative **66** was prepared on multi-gram scale from commercially available 6-hydroxyindole.^{63,83,91} Key steps included a Danishefsky 9-BBN reverse-prenylation of 3-chloroindole species **59** (ref. 92 and 100) and Claisen-based rearrangement of the propargyl ether **62** to the requisite pyran **63**. Somei–Kametani^{93–96} gramine-based construction of the amino acid and protecting group adjustment furnished the key tryptophan derivative **66** that has been deployed in numerous total syntheses of the stephacidins and notoamides.

As described in Scheme 20, β -hydroxyproline ethyl ester **67** was coupled with tryptophan species **66** employing peptide coupling reagent BOPCl. This provided the incipient dipeptide, which was then subjected to removal of the *N*-Fmoc residue allowing spontaneous cyclization to dioxopiperazine **68**. Mitsunobu dehydration directly led to the tautomerized azadiene species **8**,⁹⁷ that was stable enough to be isolated by PTLC and observable by ¹H NMR, but spontaneously underwent IMDA cycloaddition to give stephacidin A and C6-*epi*-stephacidin A as a 2.4 : 1, *syn* : *anti* mixture in 64% combined yield.⁶⁷

Oxidation of synthetic stephacidin A with the Davis saccharin-derived oxaziridine (**69**)⁹⁸ directly provided the spiro-oxindole product, notoamide B.⁶⁷ Asymmetric syntheses of stephacidins A, B, and notoamide B were reported in 2007, utilizing a facially-controlled intramolecular S_N2' reaction.⁹⁹

Notably, (–)-stephacidin A was achieved in 17 steps with a 6% overall yield. (+)-Stephacidin B is accessed from (–)-stephacidin A totaling a synthesis of 19 steps with a 1% overall yield. Additionally, (+)-notoamide B can be synthesized from the (–)-enantiomer of stephacidin A *via* the single oxidative pinacol step in 65% yield.⁹⁹

Grubbs, *et al.*, have also been able to conscript the oxaziridine oxidation to a short, biomimetic synthesis of notoamide C, C3-*epi*-notoamide C and notoamide D as shown in Scheme 21.⁶⁶ Using the same reverse-prenylated tryptophan species **66**, coupling with (*S*)-proline and cyclization provided a diastereomeric mixture of dioxopiperazines **72** and **73** that were separated and oxidized with the Davis oxaziridine to provide notoamide C (28%), C3-*epi*-notoamide C (48%) and a small amount of notoamide D (together with the corresponding 2,3-epimer in 10% combined yield).⁶⁶

Similarly, notoamide J was prepared as detailed in Scheme 22 deploying the same Davis oxaziridine oxidation of indole **76** to provide notoamide J as a 2 : 1 diastereomeric mixture.^{92,100}

As detailed in Scheme 23, biomimetic total syntheses of the malbrancheamides have been successfully realized, deploying the same approach. In contrast to the stephacidin substrate, the Mitsunobu dehydration stops at the stage of dehydroproline derivative **81** that has to be treated with aqueous KOH in methanol at room temperature or below to effect tautomerization to the requisite azadiene that then suffers IMDA cycloaddition to give the cycloadducts **83** and **84** in a ~1 : 2 *anti* : *syn* diastereomeric ratio in good overall yields.⁸⁸ Reduction of the tertiary amide in the presence of the secondary amide was readily achieved using di-isobutyl aluminum hydride.⁸⁸

This same approach was also successfully deployed to effect a concise, biomimetic total synthesis of marcfortine C as illustrated in Scheme 24.⁹⁰ Readily available β -hydroxypipelicolic acid was coupled to reverse-prenylated tryptophan derivative **66** and carried through the analogous sequence deployed for the stephacidin A synthesis. The final *spiro*-oxindole oxidation required “protecting” the tertiary amine as the corresponding tosylate salt, which permitted smooth and diastereoselective oxidation and *spiro*-rearrangement to D,L-marcfortine C. This synthesis required a total of fifteen steps from commercially available 6-hydroxyindole with an overall yield of 3.7%.⁹⁰

The asymmetric biomimetic total synthesis of versicolamide B has also been achieved and is described in Scheme 25.^{101,102} Similar to the previous discussion, pinacol rearrangement of reverse prenylated indole **68** allows for formation of a set of *spiro*-oxindole diastereomers. Upon separation, they can consequently be converted into both enantiomers of versicolamide B *via* IMDA cycloaddition. The C3-epimer of (+)-versicolamide B, recently isolated by the Tsukamoto laboratories from *A. taichungensis* and now denoted as *ent*-Taichunamide E,³⁰ has thus also been achieved *via* this synthetic route (Scheme 25).

Synthetic, isotopically labeled samples of notoamide T and 6-*epi*-notoamide T were prepared by the biomimetic IMDA strategy as shown in Scheme 26.⁶³ The doubly ¹³C-labeled samples of notoamide T and 6-*epi*-notoamide T were deployed in the precursor incorporation experiments described above (Scheme 9, Fig. 8) revealing that 6-*epi*-notoamide T is the biosynthetic precursor to versicolamide B and 6-*epi*-stephacidin A and provided the substrates from which the hitherto unobserved new metabolites were constructed from dormant tailoring genes.^{62–64} Efforts are underway to prepare optically pure samples of the individual enantiomers of 6-*epi*-notoamide T to more deeply interrogate the triggering mechanism for the expression of the dormant tailoring genes responsible for the biosynthesis of these new structures which were only produced in the presence of synthetic, racemic 6-*epi*-notoamide T.

For studies relevant to the biosynthesis of the paraherquamides, the IMDA strategy has also been applied to complete the total syntheses of both racemic and optically pure VM55599 and diastereomeric congeners. As shown in Scheme 27, optically pure VM55599, a metabolite co-produced with paraherquamide A, was prepared by the IMDA cycloaddition of *O*-acyl azadiene species **111** generated under conditions reported by Liebscher.⁵⁷

This synthesis enabled the confirmation of the absolute configuration of (–)-VM55599 as the original isolation of this natural, trace metabolite only assigned the relative

configuration. As Stocking, *et al.* had demonstrated by labeled precursor incorporation experiments that the biogenetic source of β -methylproline in paraherquamide A was (*S*)-isoleucine,¹⁰³ they had tentatively assigned the absolute stereostructure shown (Scheme 27).^{104–106} Culturing *Penicillium fellutanum* in a large format enabled the isolation of about 0.5 mg of natural (–)-VM55599.¹⁰⁷ The synthetic material obtained, which was also derived from (*S*)-isoleucine *via* the Hoffman–Loeffler–Freitag protocol, exactly matched the natural species.⁵⁷ Of additional significance, diastereomer **112** was not detected as a reaction product from this laboratory IMDA cycloaddition, revealing that the intrinsic facial bias of this particular IMDA construction favors the VM55599 stereochemistry and not the paraherquamide^{108–111} stereochemistry. This provides corroborating, indirect evidence that the enzyme responsible for the key cycloaddition that constructs the bicyclo[2.2.2]diazaoctane core in paraherquamide biosynthesis, must organize the conformation of the putative azadiene (presumably the unacylated congener of **111**) to favor the paraherquamide stereochemistry in both a highly diastereoselective and enantioselective fashion. Stocking, *et al.*, have also developed a racemic, biomimetic synthesis of VM55599 and the same group of diastereomeric congeners corresponding to **112–114** as illustrated in Scheme 28.^{58,112} In this instance, all four diastereomers were produced in the IMDA cycloaddition *via* **121**, which permitted the preparation of an authentic specimen of pre-paraherquamide by diisobutyl aluminum hydride reduction of **122**. As both amino acid subunits **47** and **116** were prepared from glycine, this synthesis was adopted to prepare double ¹³C-labeled versions of **122–125**, VM55599 and pre-paraherquamide that were used in precursor incorporation experiments.¹¹²

5. The dioxopiperazine/monooxopiperazine quandary

It was initially assumed that the bicyclo[2.2.2]diazaoctane ring systems that are common to the monooxopiperazine natural alkaloids, that includes the paraherquamides, marcfortines, asperparalines,^{113,114} and malbrancheamides, proceeded *via* biogenetic paths analogous to that of the dioxopiperazine families described in detail above, wherein a late-stage reduction of the tryptophan-derived carbonyl group was presumed to occur after the IMDA construction. This assumption has proven to be incorrect based on the following observations. As shown in Scheme 29, precursor incorporation studies with racemic, doubly ¹³C-labeled **126**, **127**, VM55599 and pre-paraherquamide were conducted in cultures of *Penicillium fellutanum*, and only pre-paraherquamide incorporated into the paraherquamide A isolated from this experiment (0.73% incorporation).¹¹² In particular, the observed lack of incorporation of **126** was puzzling, which was presumed as the biosynthetic precursor to pre-paraherquamide.

Ding, *et al.*, observed exactly the same phenomena in malbrancheamide biosynthesis as shown in Scheme 30. Again here, the biomimetic synthesis of pre-malbrancheamide to prepare doubly ¹³C-labeled isotopomers of **128** and premalbrancheamide was adopted.¹¹⁵ When both substrates were presented to cultures of *Malbranchea aurantiaca*, only premalbrancheamide incorporated into malbrancheamide B (5.5% incorporation). The lack of incorporation of either dioxopiperazine species **126** and **128** begged the question as to

when and how the tryptophan-derived carbonyl group is reduced by a net four-electrons? The answer to this quandary came in the most elegant way.

When the NRPS modules from the paraherquamide and malbrancheamide biosynthetic gene clusters were sequenced, it was revealed that the tryptophan module contains a terminal reductase domain, as opposed to the condensation domains evident in both amino acid-loading NRPS modules in notoamide biosynthesis. This is illustrated in Scheme 31.⁶² Thus, Li, *et al.*, have devised a new biogenetic hypothesis that envisions reductive release of the pro-trp dipeptide from the NRPS module, by either a two-electron or four-electron process (the four-electron reduction is shown) providing species **131** (or, the corresponding aldehyde if the reductive release is a two-electron process) which is then reverse-prenylated and oxidized (for **131**; or just reverse-prenylated for the two-electron process) to give key amino-aldehyde intermediate **132** that should spontaneously cyclize to carbinolamine **133**. Subsequent dehydration to **134** and tautomerization to azadiene species **135** would then allow for IMDA cycloaddition, directly providing pre-paraherquamide (R = Me) and pre-malbrancheamide (R = H), respectively.⁶² This proposed biogenetic pathway explains why the dioxopiperazine species **126** and **128** did not incorporate as they are not pathway intermediates!

The gene clusters for the post-NRPS module tailoring enzymes that construct species **132** merits additional scrutiny as the timing of the events leading from the NRPS module to the cycloadducts remains to be resolved.

6. Fungal producers of prenylated indole alkaloids

A large number of species of *Penicillium* and *Aspergillus*, both presently with approximately 400 described and accepted species^{116–119} are able to produce prenylated indole alkaloids, including penitrems, cyclopiazonic acid, aflavinins, paspalines fumigaclavines, janthitrems/shearinins, thiersindoles, fumitremorgins, roquefortines, rugulovasines *etc.*^{120,121} In *Penicillium*, these secondary metabolites are predominantly produced by species in subgenus *Penicillium*, but several producers are also found in subgenus *Aspergilloides*. While penitrems are restricted to subgenus *Penicillium*, the related shearinins are produced by species in both subgenera. Several species in *Penicillium* produce roquefortine C as the biosynthetic end-product, including *P. expansum*, *P. griseofulvum*, *P. crustosum*, *P. hordei*, *P. robsamsonii*, *P. samsonianum*, *P. roqueforti*, *P. carneum* and *P. paneum*, while other species accumulate meleagrins and oxalines, derived from roquefortine C, for example *P. chrysogenum*, *P. rubens*, *P. oxalicum*, *P. compactum*, *P. concentricum*, *P. coprobium*, *P. glandicola*, *P. crystallinum* and *P. malodorum*.^{117,120,122} Interestingly, neoxaline is also produced by several species in *Aspergillus* section *Nigri*, but only in the uniseriate species.^{123,124} None of these secondary metabolites have been found outside the genera *Aspergillus* and *Penicillium*, except that few species in *Talaromyces* can produce rugulovasines.^{116,125}

The bicyclo[2.2.2]diazaoctane ring system containing prenylated indole alkaloids are more restricted in their distribution, but again most of them are produced by species of *Penicillium* and *Aspergillus*. The known exception is the malbrancheamides produced by *Malbranchea*

species,²² a species belonging to the *Myxotrichaceae* and unrelated to the *Trichocomaceae*. Since this species already produces the *Penicillium* and *Aspergillus* metabolite penicillic acid,¹²⁶ there is a possibility that the fungus was indeed correctly identified as a *Penicillium* or *Aspergillus*. Most of these unique prenylated indole alkaloids are produced by both *Aspergillus* and *Penicillium* species except that the mangrovamides,¹²⁷ brevianamides, chrysogenamide, paraherquamides and marcfortines have only been reported from *Penicillium* species, while the asperparalines, sclerotiamides, notoamides, versicolamides, avrainvillamides, stephacidins, and taichunamides have been recorded from *Aspergillus* species. However, chemotaxonomic investigations have shown that the mangrovamides and okaramines have been found in both genera (Frisvad, unpublished data). In *Penicillium*, the brevianamides are produced only by two species in subgenus *Penicillium* section *Fasciculata* (*P. viridicatum*) and subgenus *Penicillium* section *Brevicompacta* (*P. brevicompactum*).^{120,122} The marcfortines have until now only been found in *Penicillium paneum* in subgenus *Penicillium* section *Roquefortorum*.^{120,122} The paraherquamides have been found in *Penicillium paraherquei*¹⁰ (*Penicillium* section *Lanata-Divaricata*) and *P. canescens* (*Penicillium* section *Canescentia*) (originally determined as *P. charlesii*).¹¹ Chrysogenamide was reported from *P. chrysogenum*,²⁹ but the identity of the producing organisms has not been confirmed.

The producers of the related asperparalines (aspergillimides), avrainvillamides, notoamides, sclerotiamides, stephacidins, taichunamides, and versicolamides are produced by species in *Aspergillus* subgenus *Circumdati* sections *Candidi*, *Circumdati*, and *Nigri*, and in subgenus *Nidulantes* section *Versicolores*. Species producing those metabolites in section *Candidi* include *A. subalbidus* and *A. taichungensis*.^{128,129} Species in section *Nigri* producing aspergillamides include *A. brunneoviolaceus* (*A. jiensis*).¹²⁴ In section *Circumdati* the species *A. affinis*, *A. auricomus*, *A. bridgeri*, *A. melleus*, *A. ochraceus*, *A. ostianus*, *A. pallidofulvus*, *A. persii*, *A. salwaensis*, *A. sclerotiorum*, *A. sesamicola*, *A. subramanianii*, *A. westerdijkiae* and *A. westlandensis* produce “Aspergamides”, which is a mixture of notoamides, avrainvillamides, stephacidins, and sclerotiamide.¹¹⁶ Finally, *Aspergillus* subgenus *Nidulantes* section *Versicolores* contains producers of “Aspergamides”: *A. amoenus* and *A. protuberus*,³⁵ but “Aspergamides” could not be found in any species in section *Nidulantes*.¹³⁰ Further studies will show whether the producing species in these sections of *Aspergilli* all have stereochemical diversity in their production of these compounds.

7. Conclusions

The biogenesis of prenylated indole alkaloid families containing the unique bicyclo[2.2.2]diazaoctane core, obtained from various genera of marine and terrestrial fungi has revealed a number of fascinating aspects of secondary metabolite biosynthesis. First, it is clear that the biosynthetic gene clusters responsible for directing the biosyntheses of these structurally alluring natural compounds are widely distributed in the biosphere. In particular, the orthologous pairs of marine and terrestrial fungi responsible for stephacidin and notoamide biosyntheses, have extensively interrogated the stereochemistry of these molecular frameworks in both a relative and absolute sense. A vast repertoire of tailoring genes is in evidence, and the experiments described in Scheme 9 and Fig. 8 reveal that many

dormant tailoring genes likely lie in reserve in the genomes of these complex organisms. It is particularly intriguing to ponder the speciation events that occurred at some time during the evolutionary history of these fungi that resulted in enantiomerically distinct secondary metabolite profiles. Very little is known about the adaptive advantage with which these secondary metabolites embellish the producing fungi as the biological activities of these natural alkaloids have been only superficially examined and demand further elucidation.

It is also now clear that Nature has, perhaps, “accidentally” created the bicyclo[2.2.2]diazaoctane ring system through the generation of reactive, achiral azadiene species (**138**, Scheme 32). The achiral intermediates are conformationally restricted in the chiral environment of the enzyme binding sites that generate these species, and thus orchestrates these reactive azadiene species to undergo highly enantio- and diastereoselective cycloadditions culminating in the formation of the natural substances constituted with the bicyclo[2.2.2]diazaoctane core. Our biomimetic laboratory IMDA reactions, which proceed smoothly at or below room temperature in organic or aqueous solvents, are very facile transformations that in the biochemical setting, only require pre-organization of the pre-transition state conformations.

It is further noteworthy that, in the monooxopiperazine families, the predominant relative stereochemistry is *syn*- (all known paraherquamide alkaloids in this family are *syn*-; see Fig. 1 and 4) and only a single absolute configuration is currently in evidence (with the exception of VM55599, a shunt metabolite of paraherquamide biosynthesis). To date, the only monooxopiperazine-producing fungi that construct the corresponding *anti*-diastereomeric series are *Penicillium purpurogenum* G59 (penicimutamides D and E; *P. purpurogenum* G59 has been mischaracterized and is a *Penicillium citrinum*),¹⁵ *Penicillium chrysogenum* (chrysogenamide A)²⁹ and *Penicillium citrinum* F53 (the recently described citrinalin C).¹⁴⁶ In stark contradistinction, the dioxopiperazine families display a broader range of relative and absolute stereochemistry, where a myriad of both *syn*- and *anti*-metabolites have been isolated in both enantiomeric forms. Most intriguing from an evolutionary perspective, the enantio-divergence evident in both marine- and terrestrial-producing fungi, provides for a very rich and unique opportunity to study molecular evolution of these organisms.

The application of whole-genome sequencing, bioinformatics analysis, cloning and functional expression of biosynthetic enzymes, synthesis of labeled pathway metabolites, precursor incorporation studies, structure determination of new metabolites and total syntheses of both new/undiscovered and known metabolites has been deployed to gain a deeper understanding of the secondary metabolome of marine and terrestrial fungi that produce these families of alkaloids. It should be noted that many laboratories in the synthetic community have published elegant, yet non-biomimetic total syntheses of numerous natural products in this family of alkaloids.^{41,46,47,57,58,66,67,85,86,88,90,95,97,99,102,108,115,131–146} These efforts have been reviewed by Miller and Williams in 2009 (ref. 40) and additional total syntheses have appeared in the literature since 2009.^{138–146} Complete knowledge of the structural and stereochemical homologies and disparities among these compounds continues to motivate research into the synthesis, chemistry, biochemistry, genetics and biology of natural fungal metabolites. It is anticipated that exciting new chapters of these fascinating natural alkaloids remain to be written.

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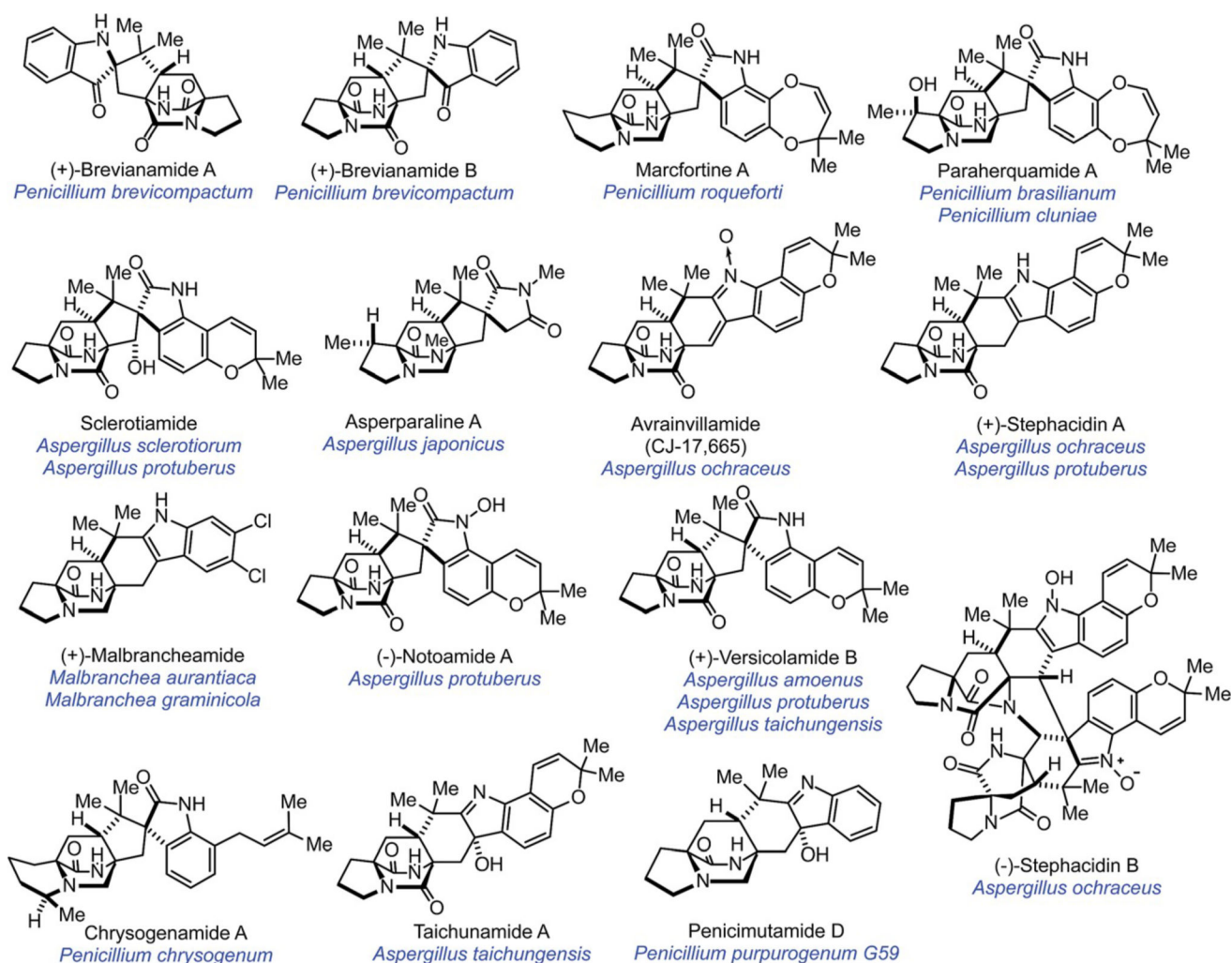


Fig. 1.
Structures of representative prenylated indole alkaloids isolated from fungi of the genera *Aspergillus*, *Penicillium*, and *Malbranchea*.

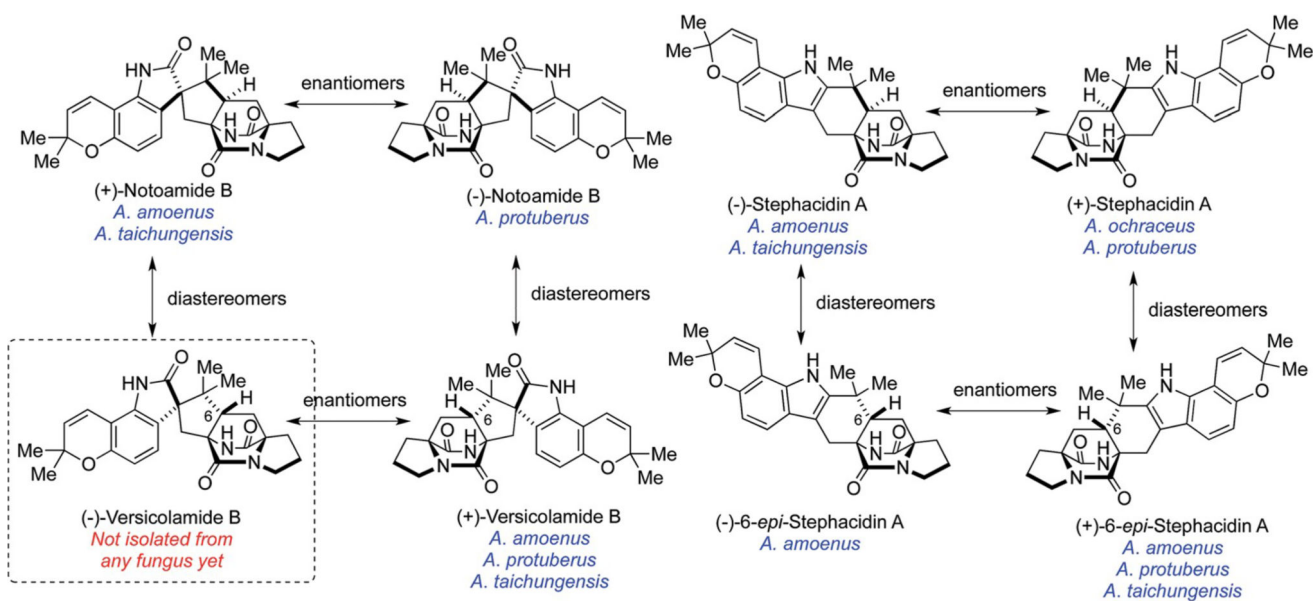
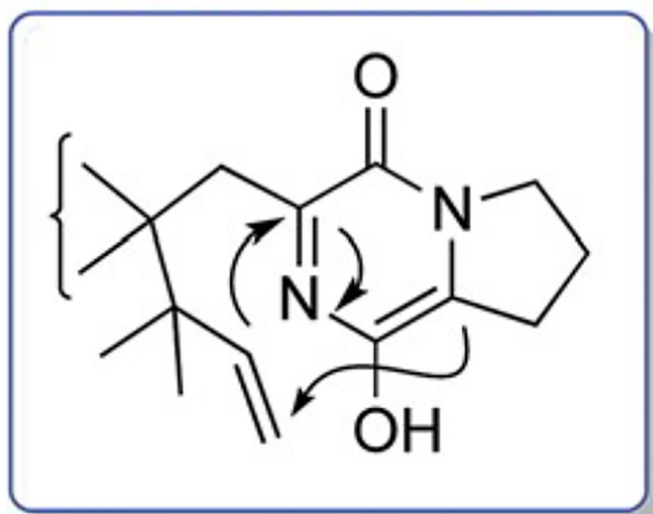


Fig. 2.
Structures of several prenylated indole alkaloids isolated from fungi and their stereochemical relationships.

Sammes, 1970



Birch, 1971

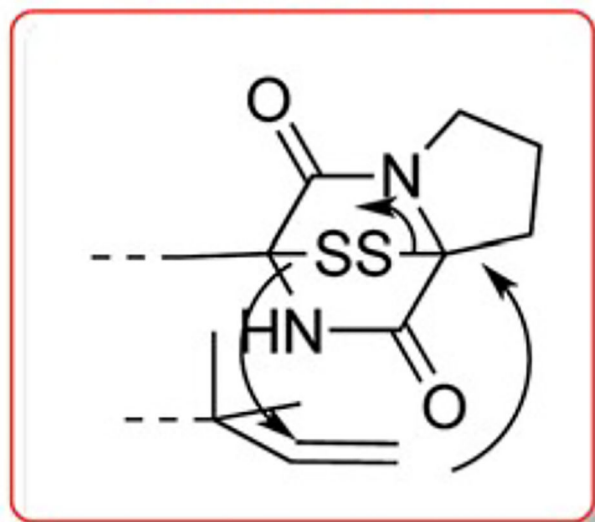


Fig. 3.
Early suggestions of Sammes⁴³ and Birch⁴⁵ concerning the biogenesis of the bicyclo[2.2.2]diazaoctane ring system in the brevianamides.

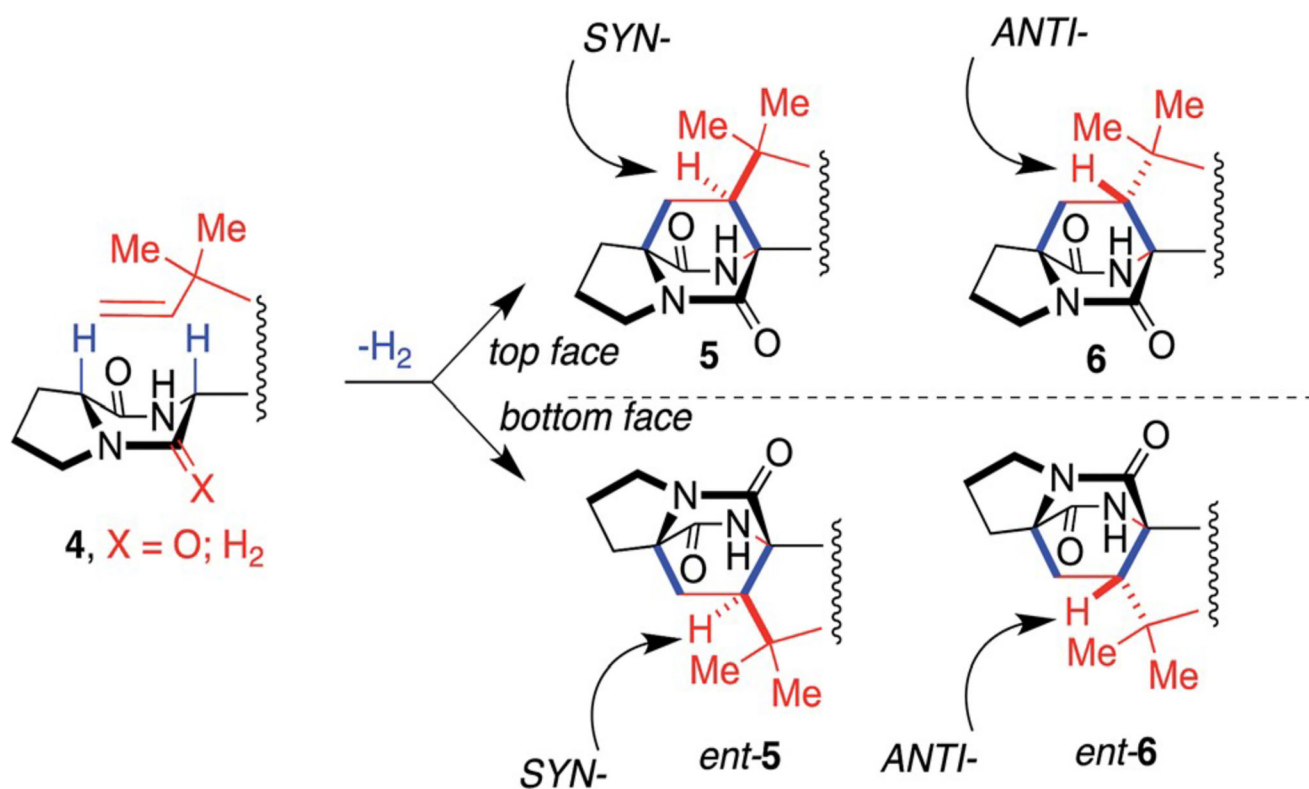


Fig. 4. Stereochemical outcomes of the oxidative isoprene cyclization across the two amino acid α -carbons in the construction of the bicyclo [2.2.2]diazaoctane.⁶²

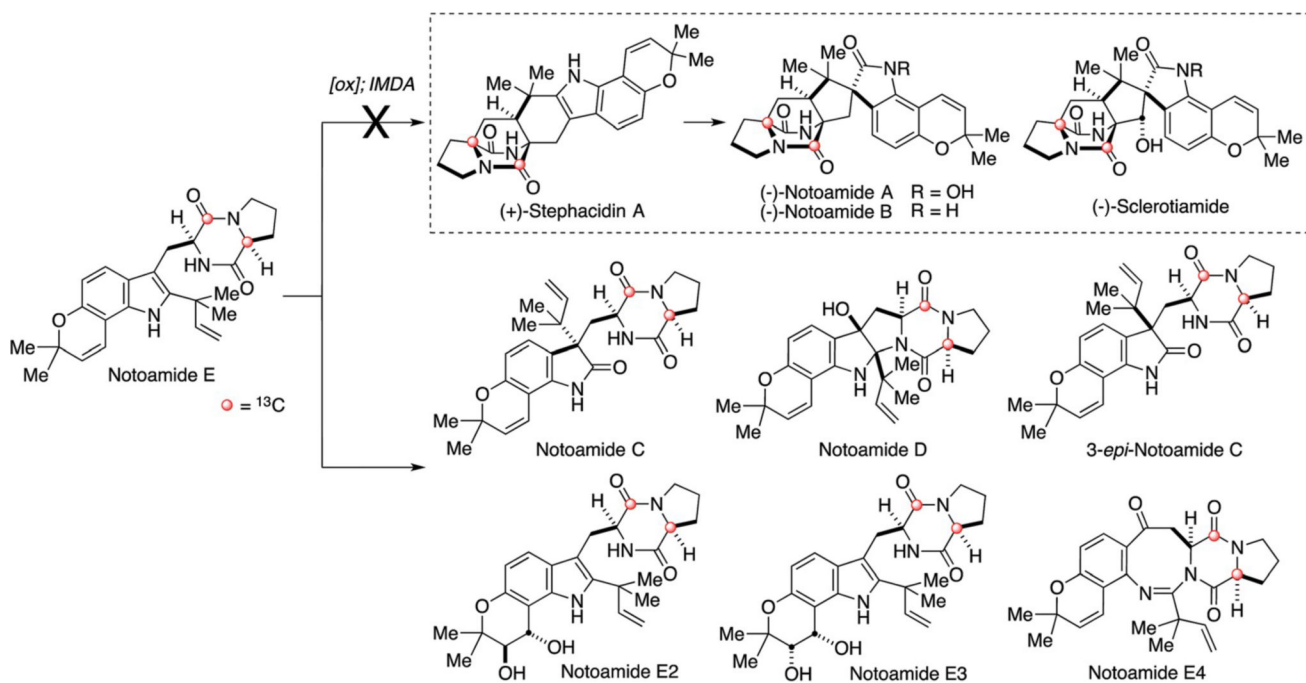


Fig. 5.
Precursor incorporation results of $[\text{C}^{13}]_2$ -notoamide E in *A. protuberus* MF297-2.⁶⁸⁻⁷¹

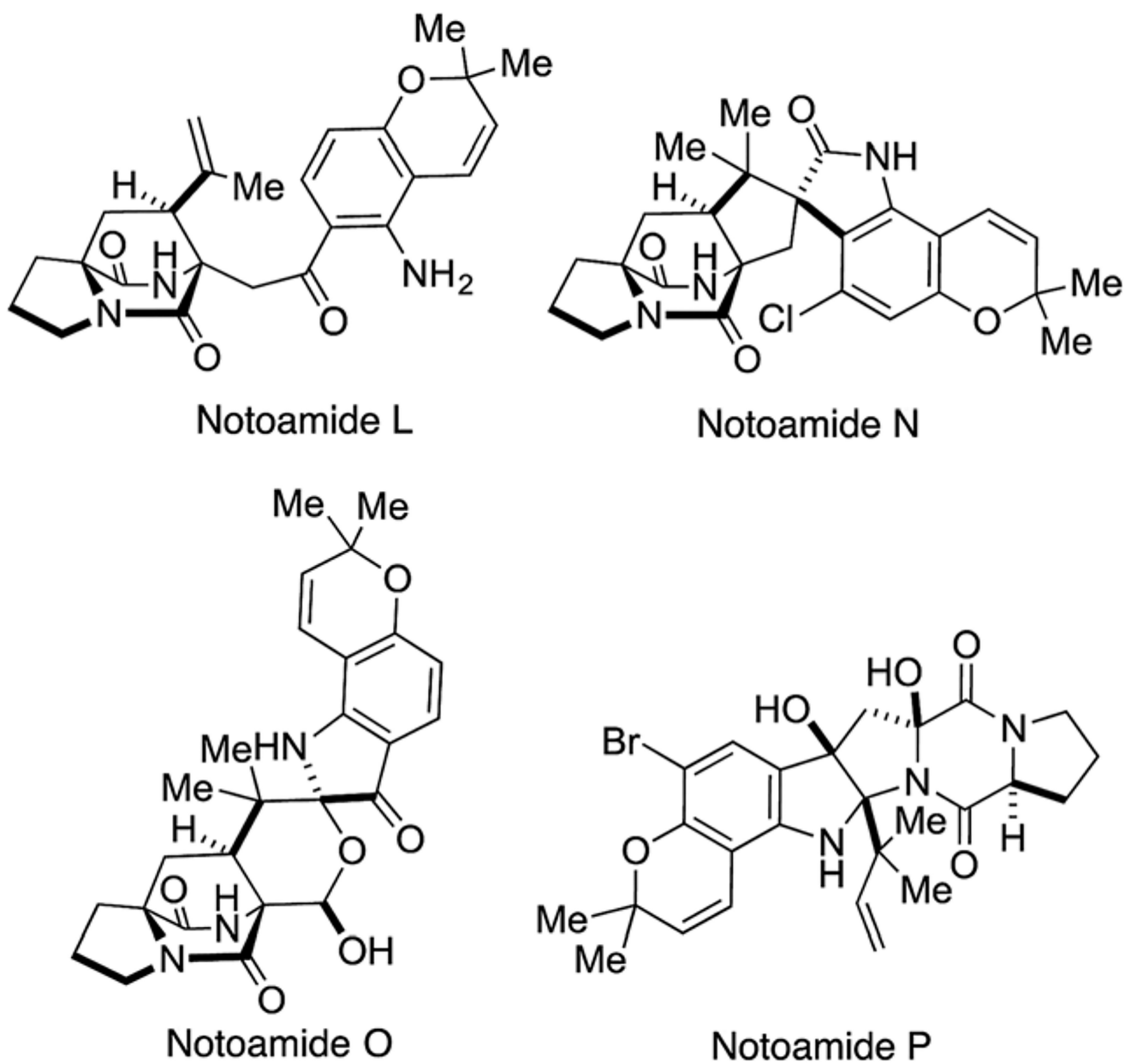


Fig. 6.
Structures of notoamides L and N-P.^{26,27,65}

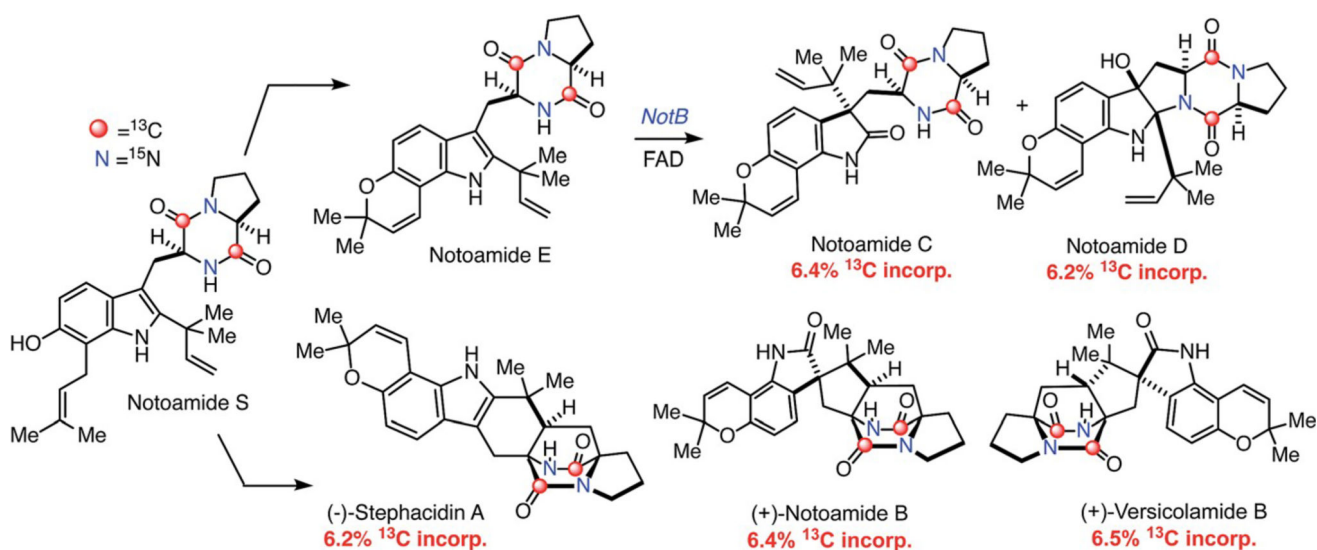
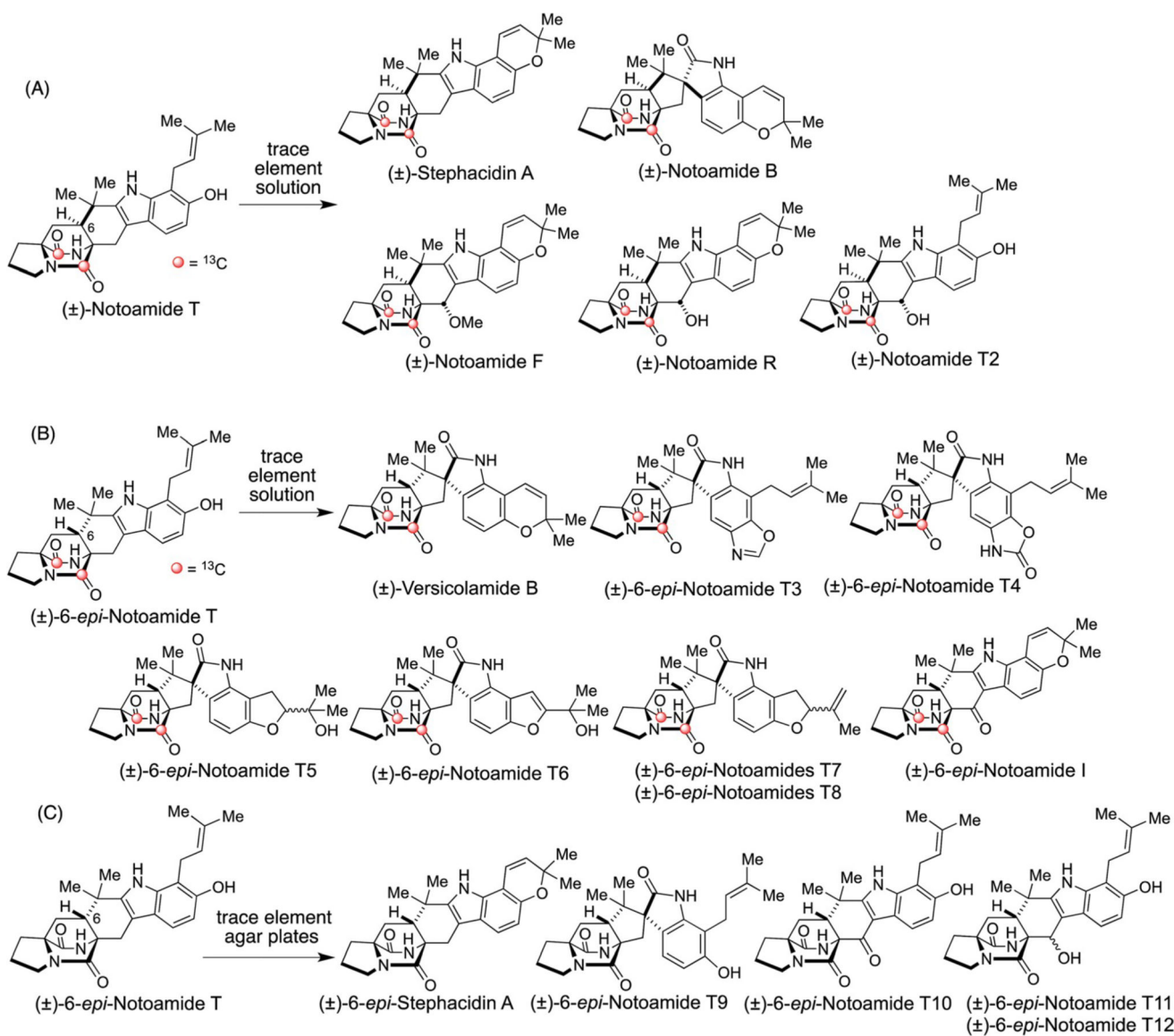


Fig. 7.
Results of the incorporation experiment of $[\text{}^{13}\text{C}]_2\text{--}[\text{}^{15}\text{N}]_2\text{--notoamide S}$ in *A. amoenus* NRRL 35660.⁷⁷

**Fig. 8.**

Results of the incorporation experiments of $[^{13}\text{C}]_2$ -(±)-notoamide T in a trace element solution (A), $[^{13}\text{C}]_2$ -(±)-6-*epi*-notoamide T in a trace element solution (B), and (±)-6-*epi*-notoamide T on trace element agar plates (C) in *A. protuberus* MF297-2.^{63,64}

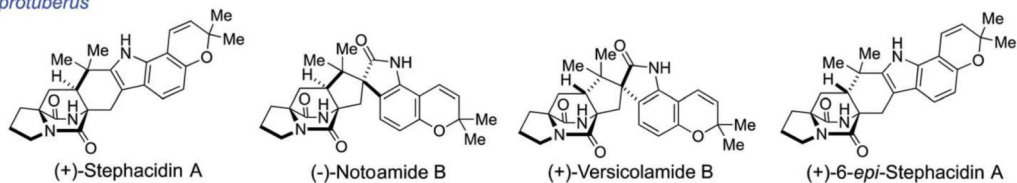
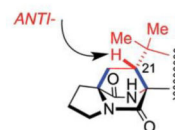
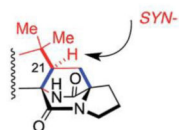
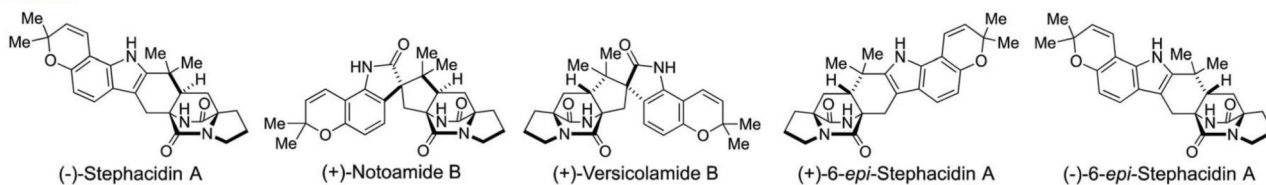
A. protuberus*A. amoenus*

Fig. 9.
Prenylated indole alkaloids illustrating the *syn*- and *anti*-diastereomeric relative configurations isolated from *A. protuberus* MF297-2 and *A. amoenus* NRL 35660.^{26,28,30,61}

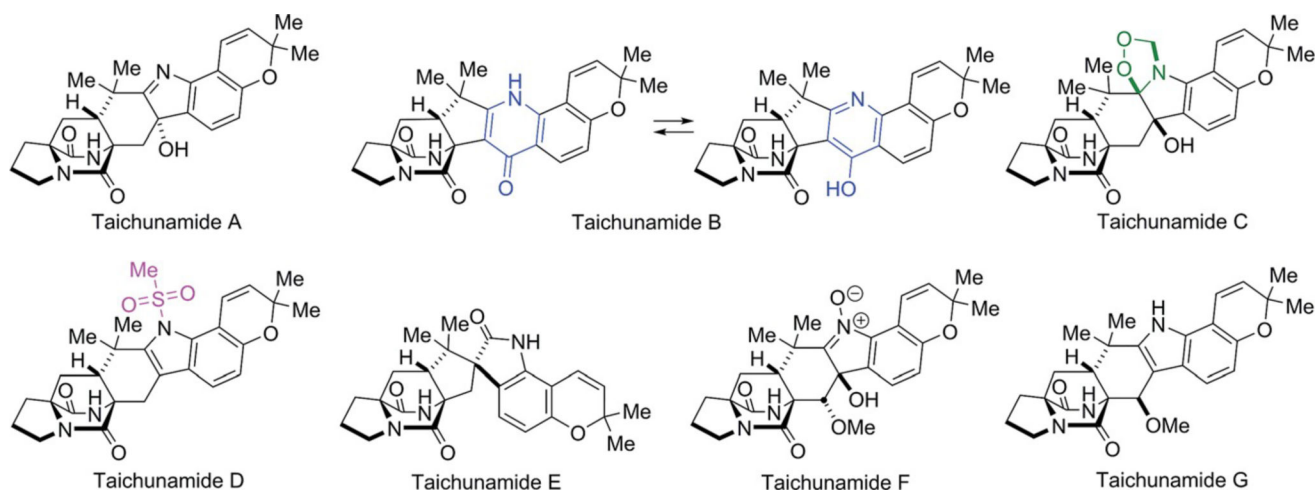
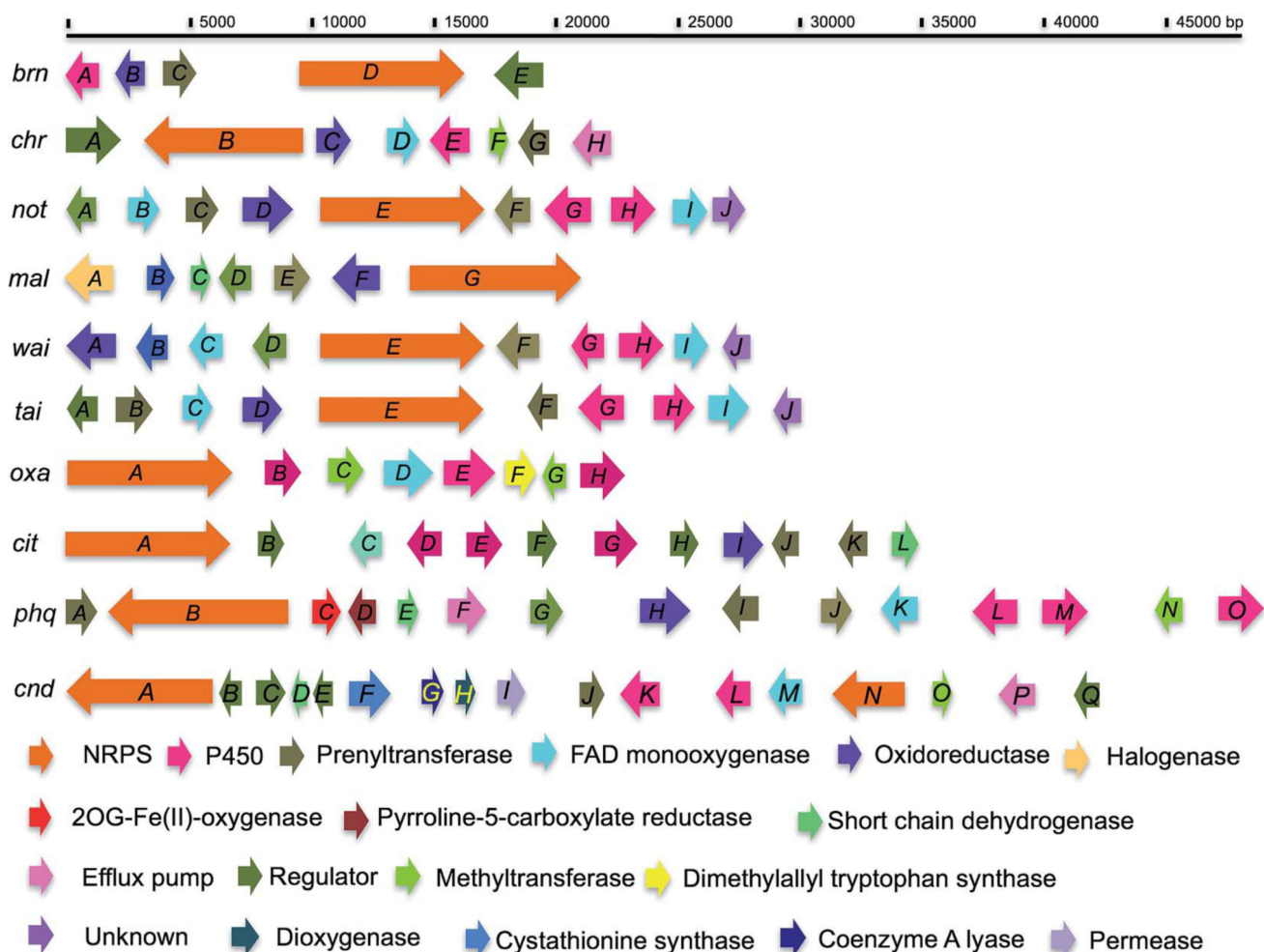


Fig. 10.
Structures of the taichunamides isolated from *A. taichungensis* IBT19404.³⁰

**Fig. 11.**

Mining and annotation of biosynthetic gene clusters of fungal indole alkaloids.

Brevianamide (*Aspergillus versicolor* MF030, brn); chrysogenamide (*Penicillium alligativum*, chr); notoamide A (*A. protuberus* MF297-2, not); malbrancheamide (*Malbranchea aurantiaca* RRC1813, mal); waikialoid A (*Aspergillus* sp., wai); 3-*epi*-notoamide C (*A. taichungensis* IBT 19404, tai); oxaline (*P. oxalicum* F30, oxa); citrinalin (*P. citrinum* F53, cit); parahequamide A (*P. fellutanum* ATCC20841, phq); citrinadins (*P. citrinum* IBT 29821, cnd).^{62,84}

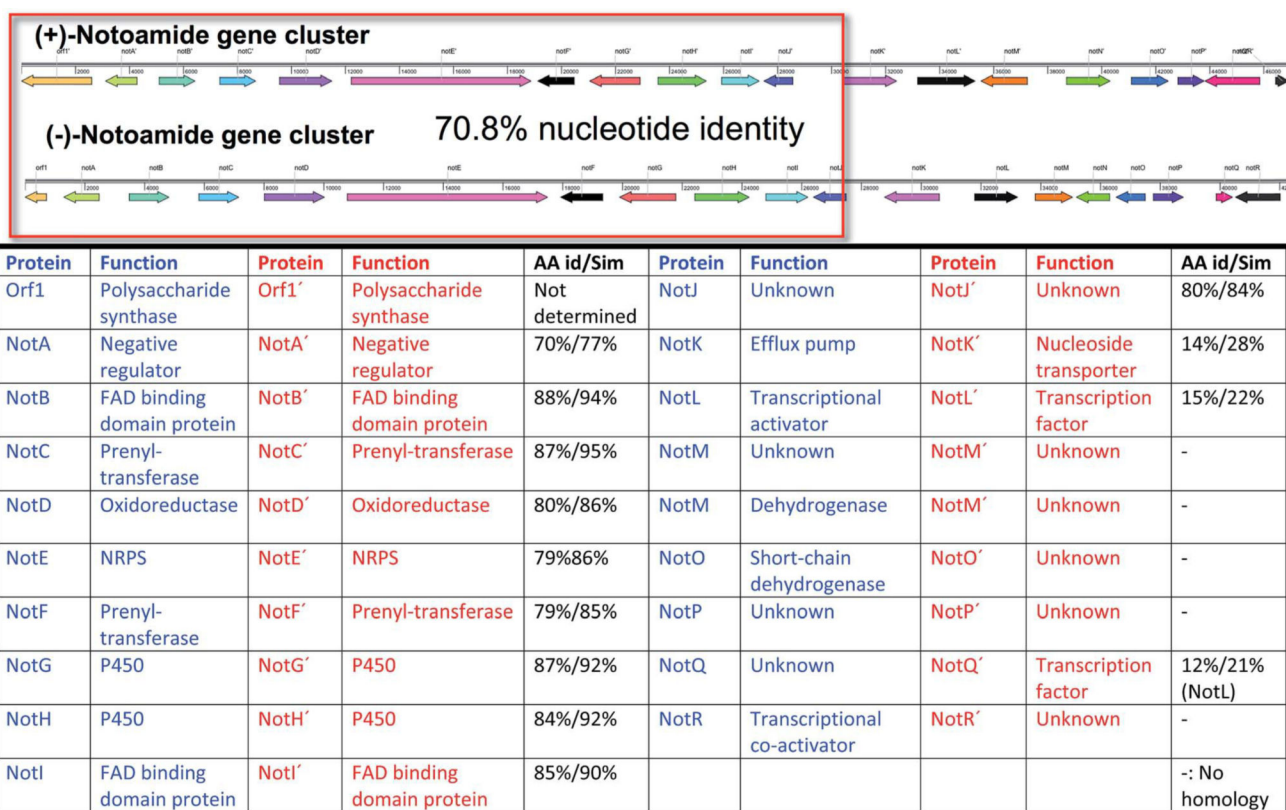
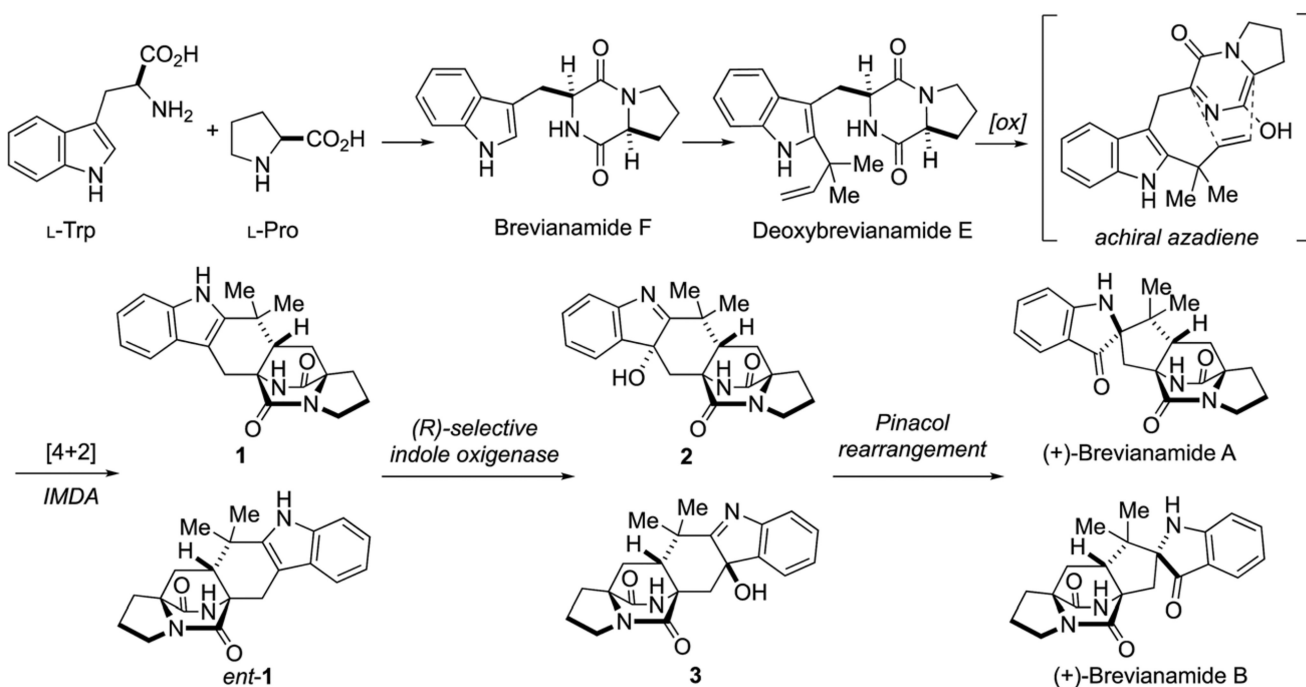
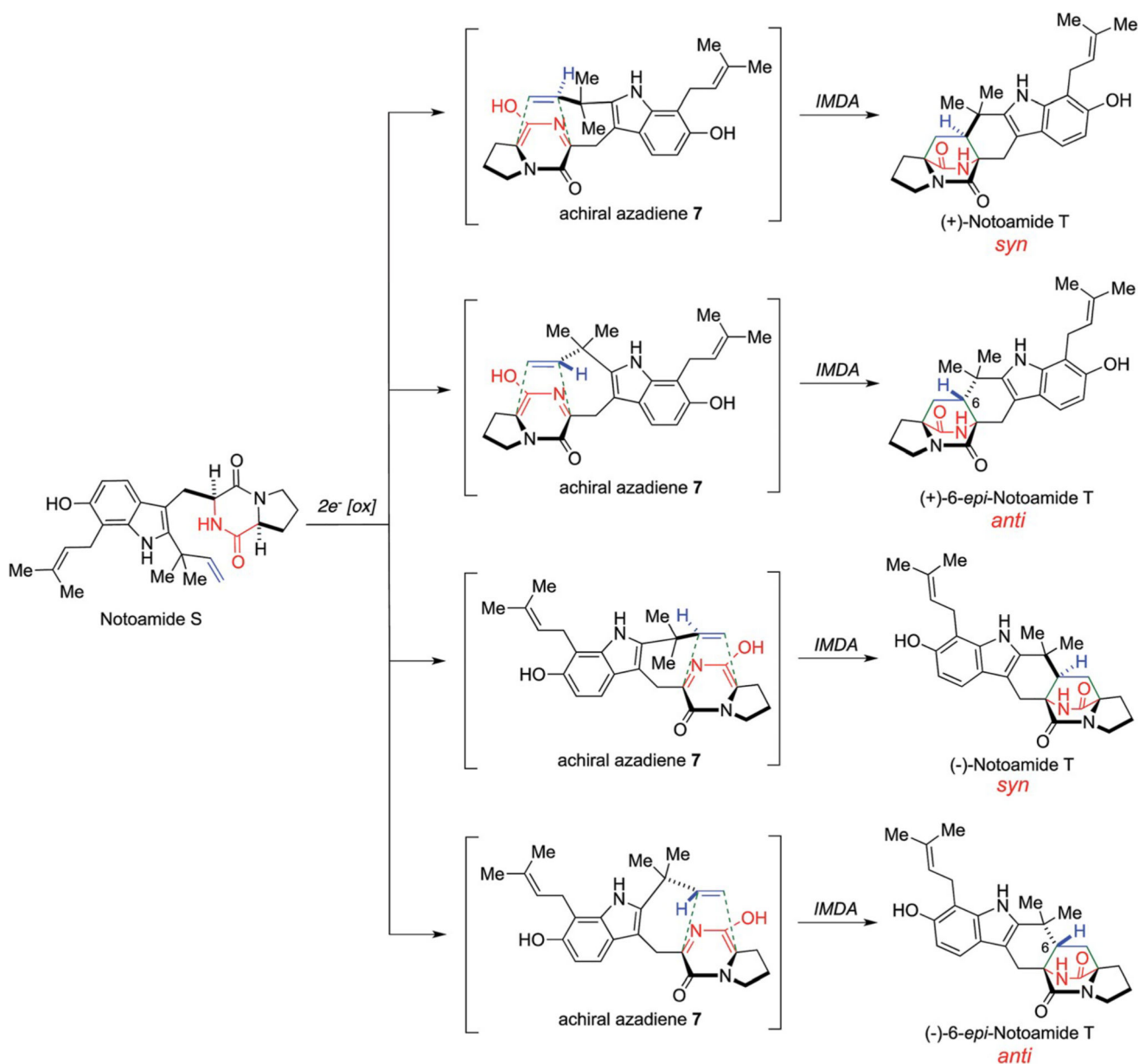
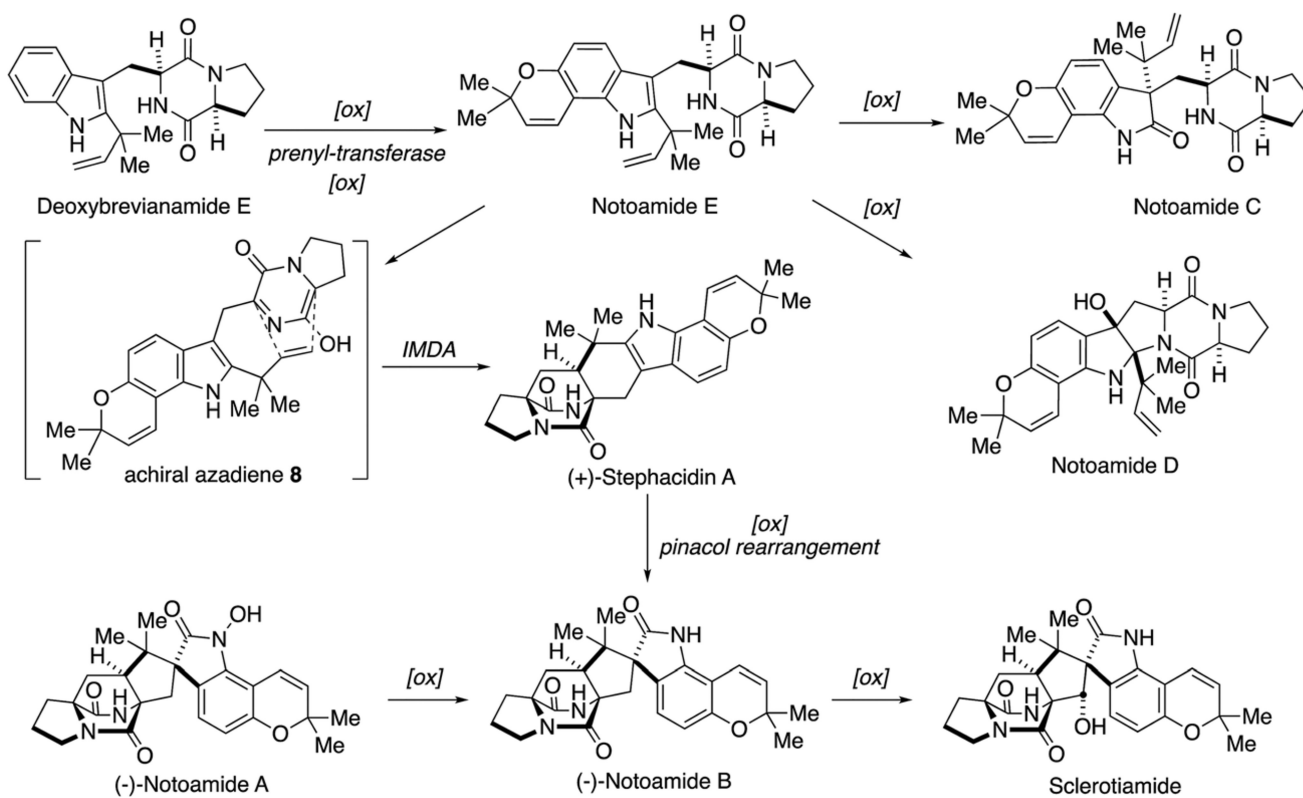


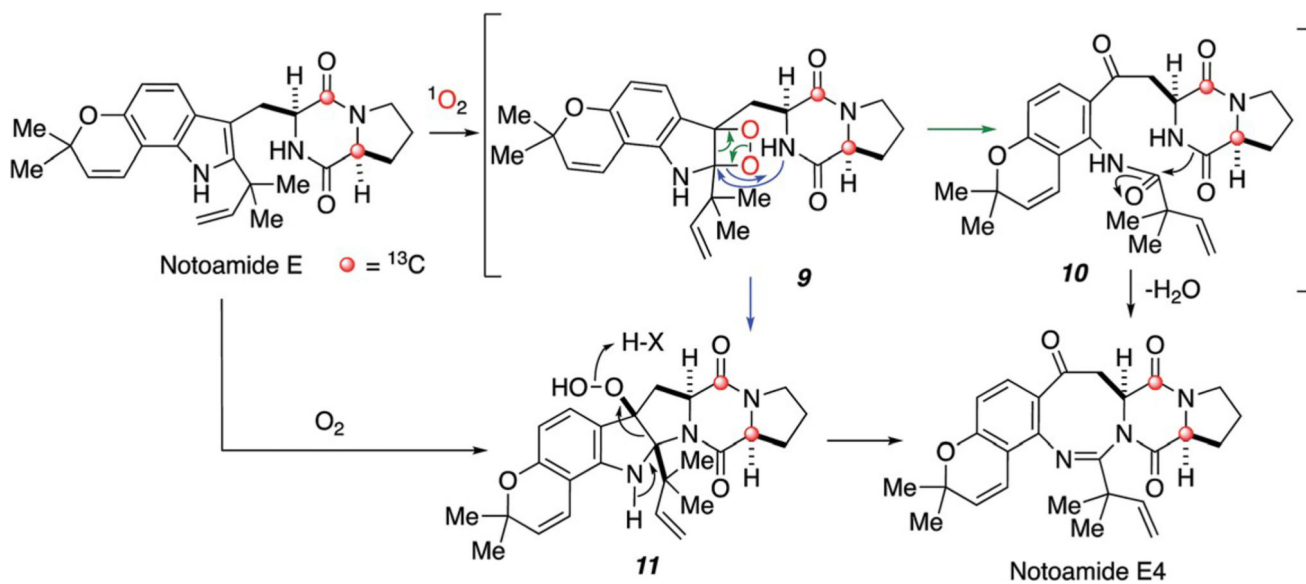
Fig. 12. Comparison of the biosynthetic gene clusters of the (+)-notoamide (*Aspergillus amoenus*) and (-)-notoamide (*Aspergillus protuberus*) producing fungi.⁸³

**Scheme 1.**Alternative brevianamide biogenesis proposed by Williams.⁴⁹

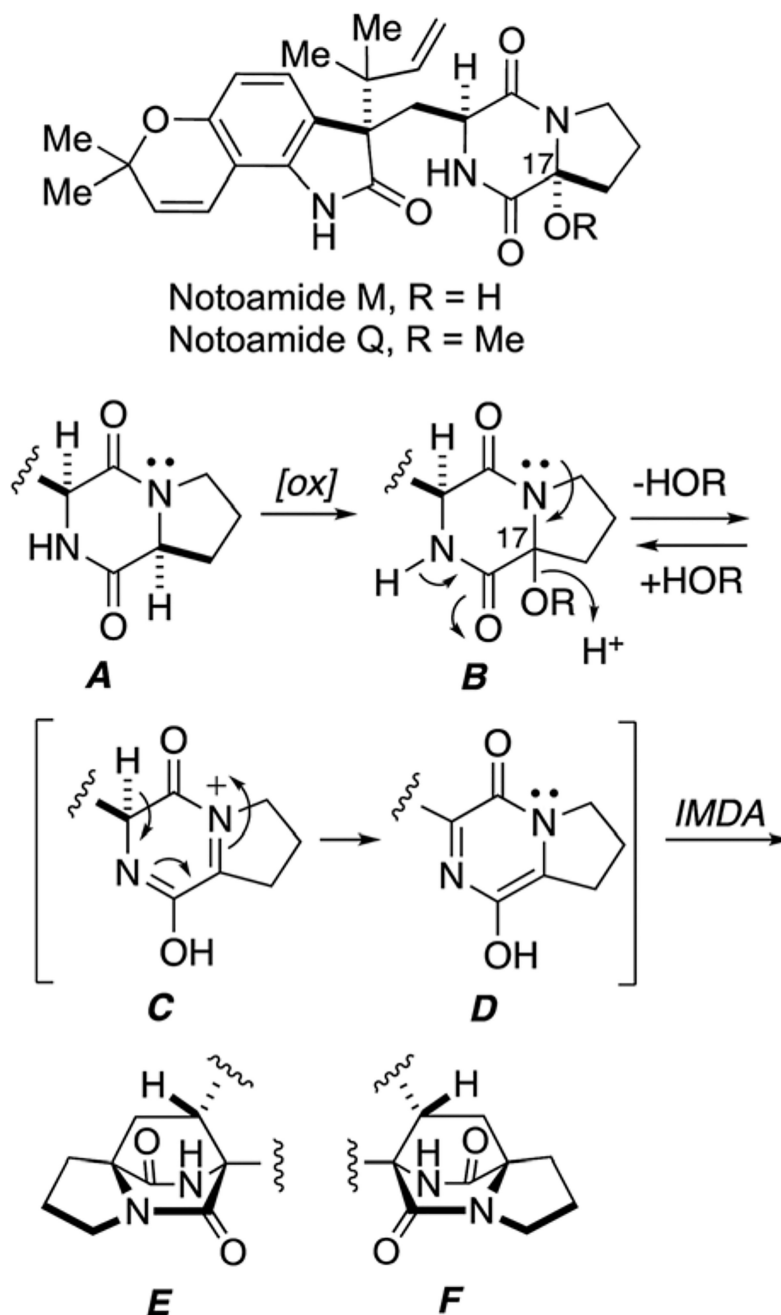
**Scheme 2.**Putative IMDA reactions in the oxidative enzymatic transformation from notoamide S.⁶¹

**Scheme 3.**

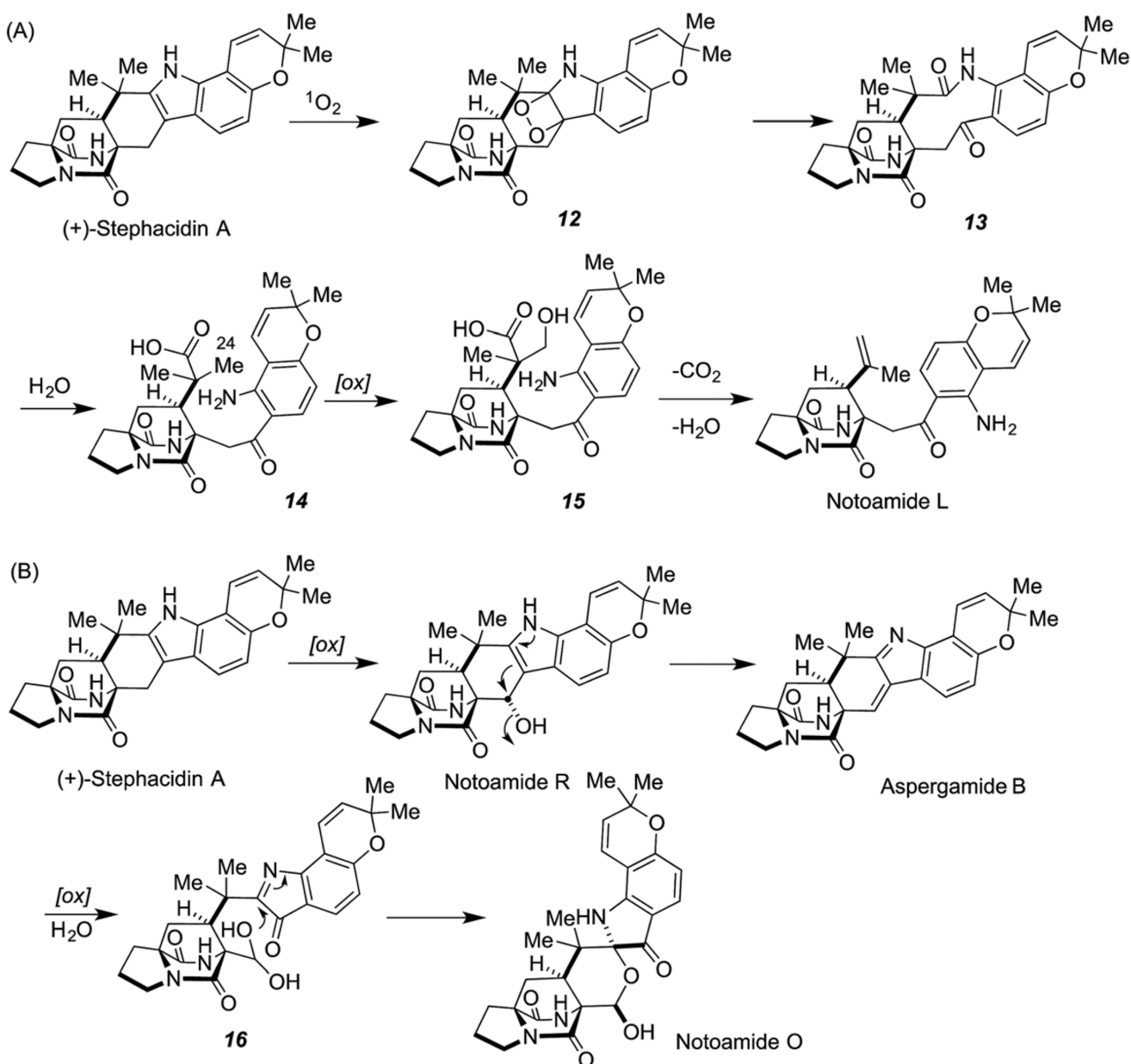
Structures of prenylated indole alkaloids isolated from *A. protuberus* MF297-2 and initial proposal regarding their possible biogenetic relationships.^{24,41,63,66–70}

**Scheme 4.**

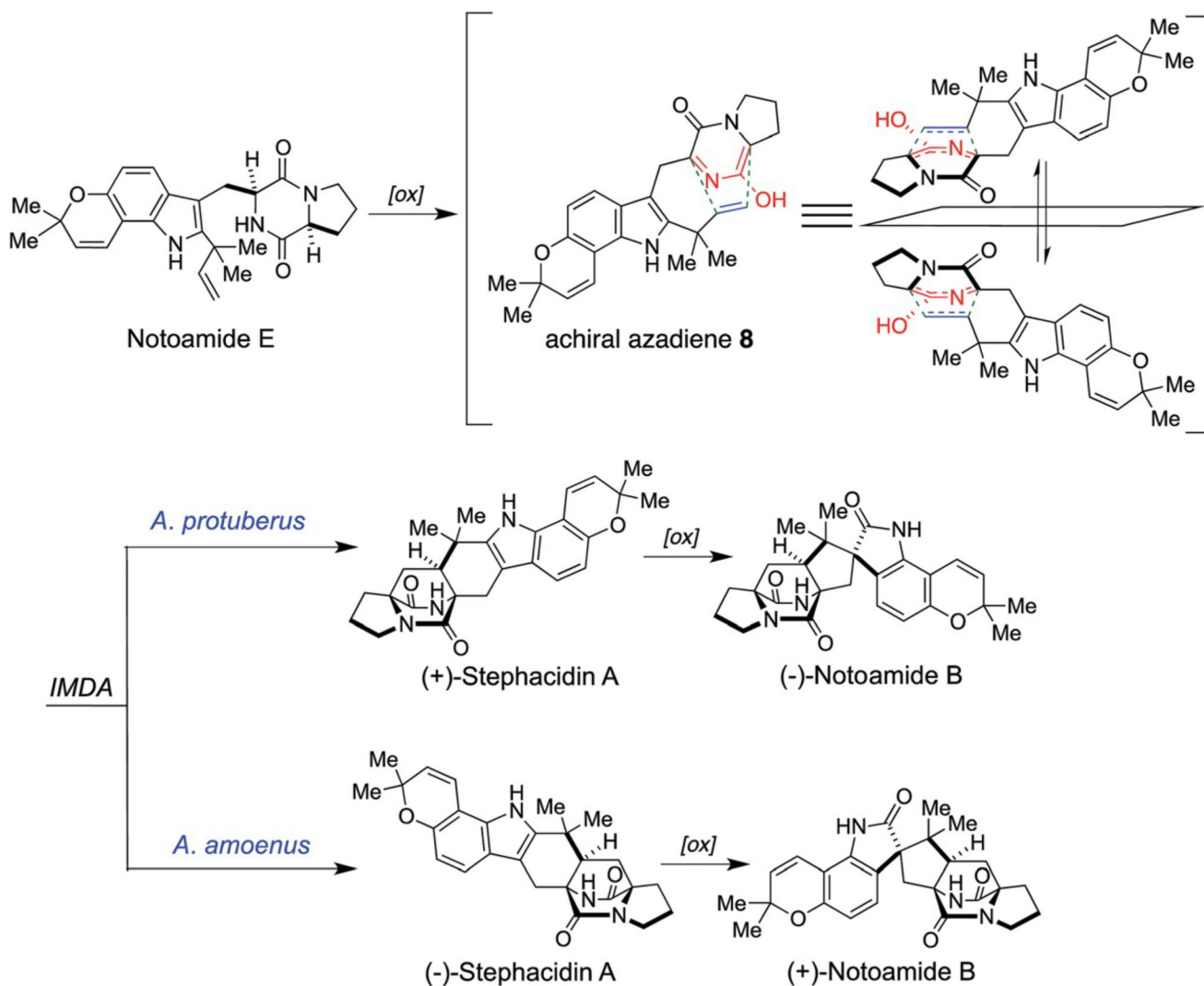
Possible biosynthetic pathways to notoamide E4 from notoamide E.⁶⁸

**Scheme 5.**

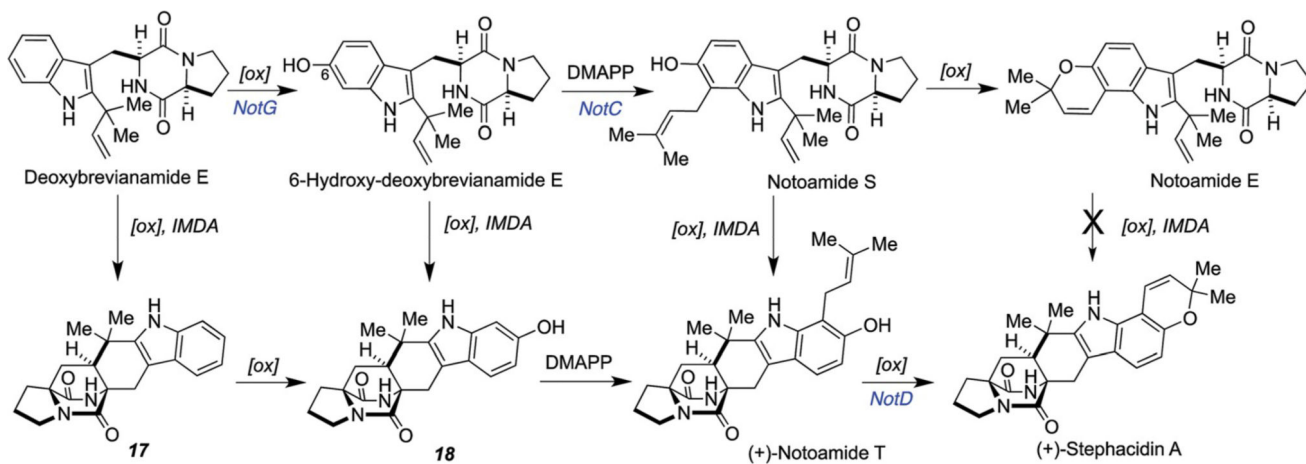
Structures of notoamides M and Q and possible formation of the bicyclo[2.2.2]diazaoctane ring system, theoretically through notoamides M and Q.^{26,27,65}



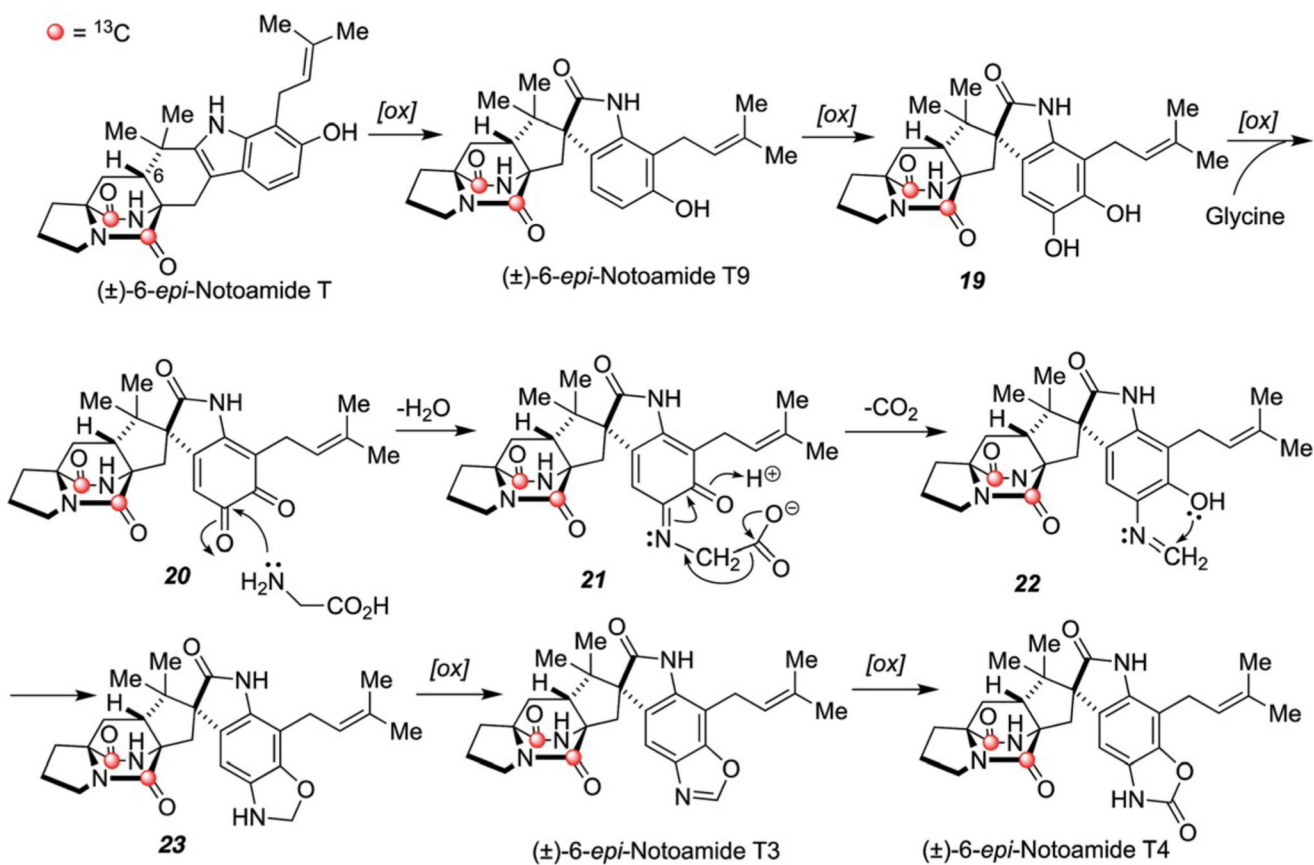
Scheme 6.
Possible biosynthetic pathways to notoamides L (A) and O (B).²⁷

**Scheme 7.**

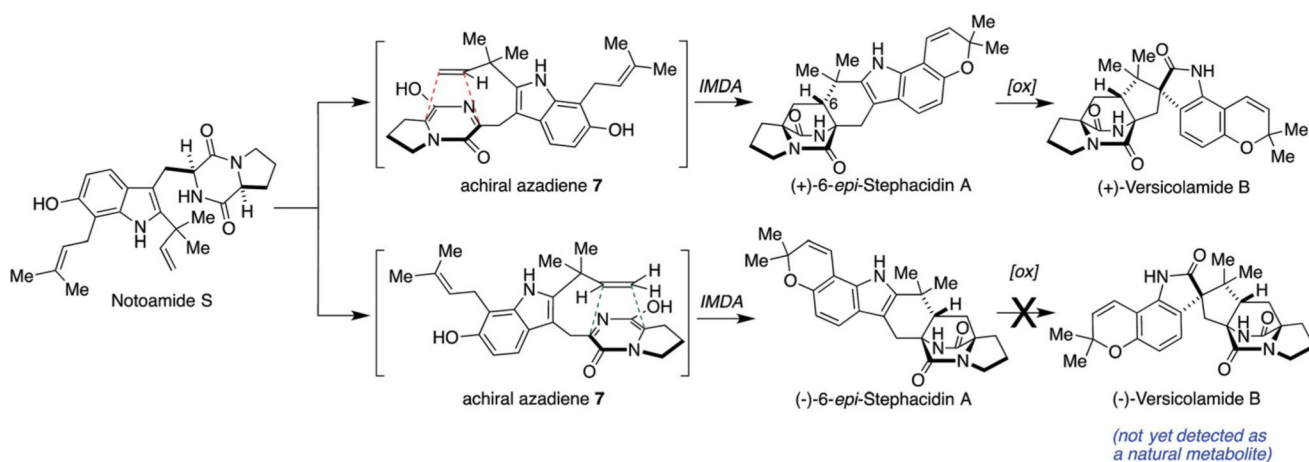
Initial proposal for the enantiodivergent biogenesis of enantiomeric prenylated indole alkaloids with a bicyclo[2.2.2]diazaoctane core in *A. protuberus* MF297-2 and *A. amoenus* NRRL 35660.^{28,70}

**Scheme 8.**

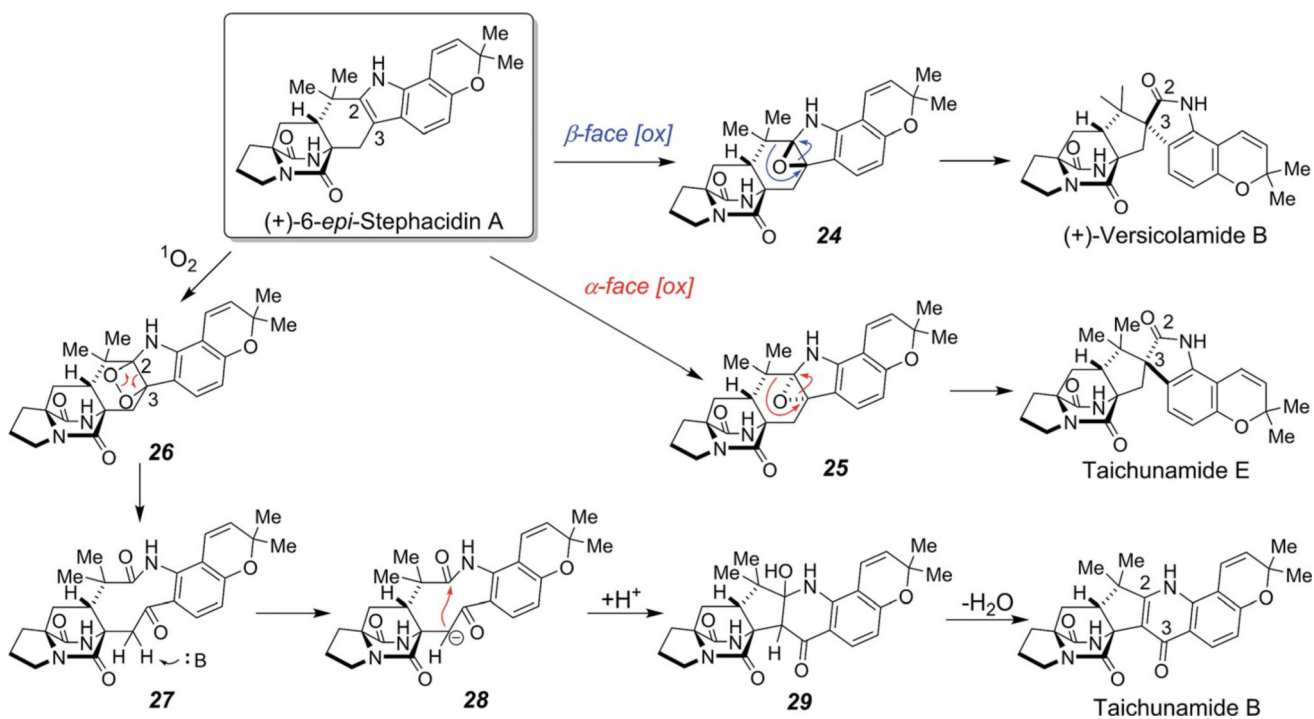
Possible biosynthetic pathways from deoxybrevianamide E to stephacidin A and potential precursors for IMDA reactions.^{74–76}

**Scheme 9.**

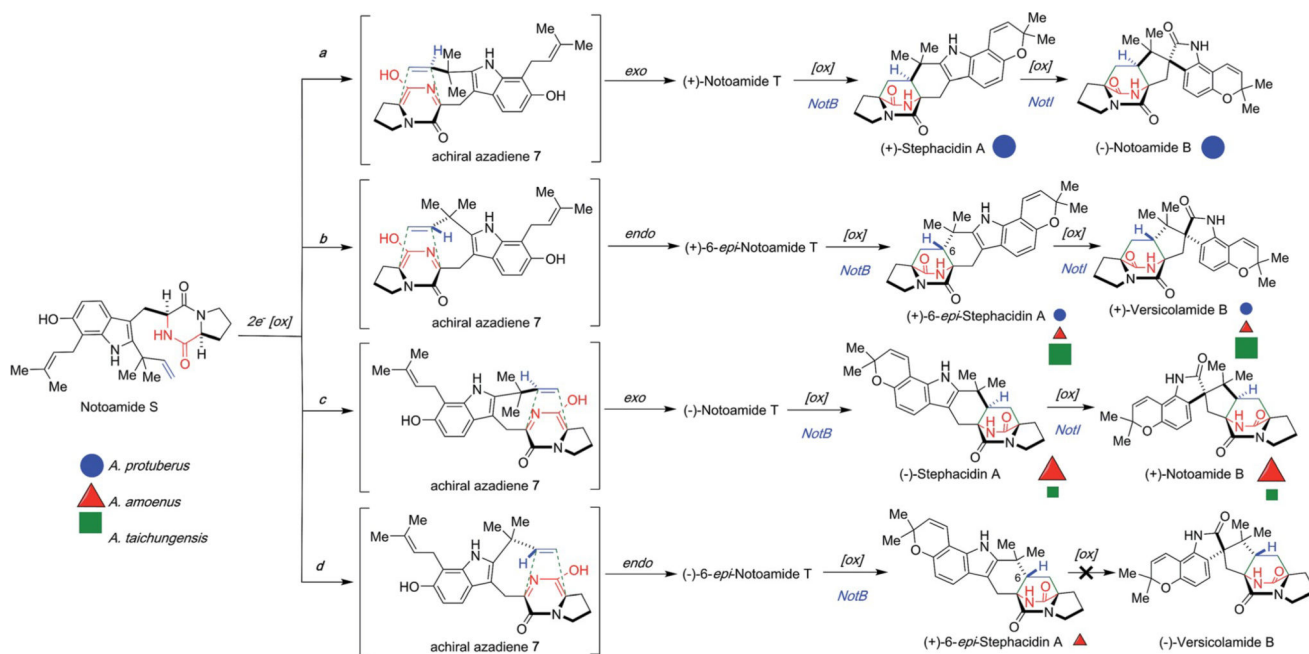
Possible biosynthetic pathways to 6-*epi*-notoamide T3 and 6-*epi*-notoamide T4 from 6-*epi*-notoamide T.^{62,64}

**Scheme 10.**

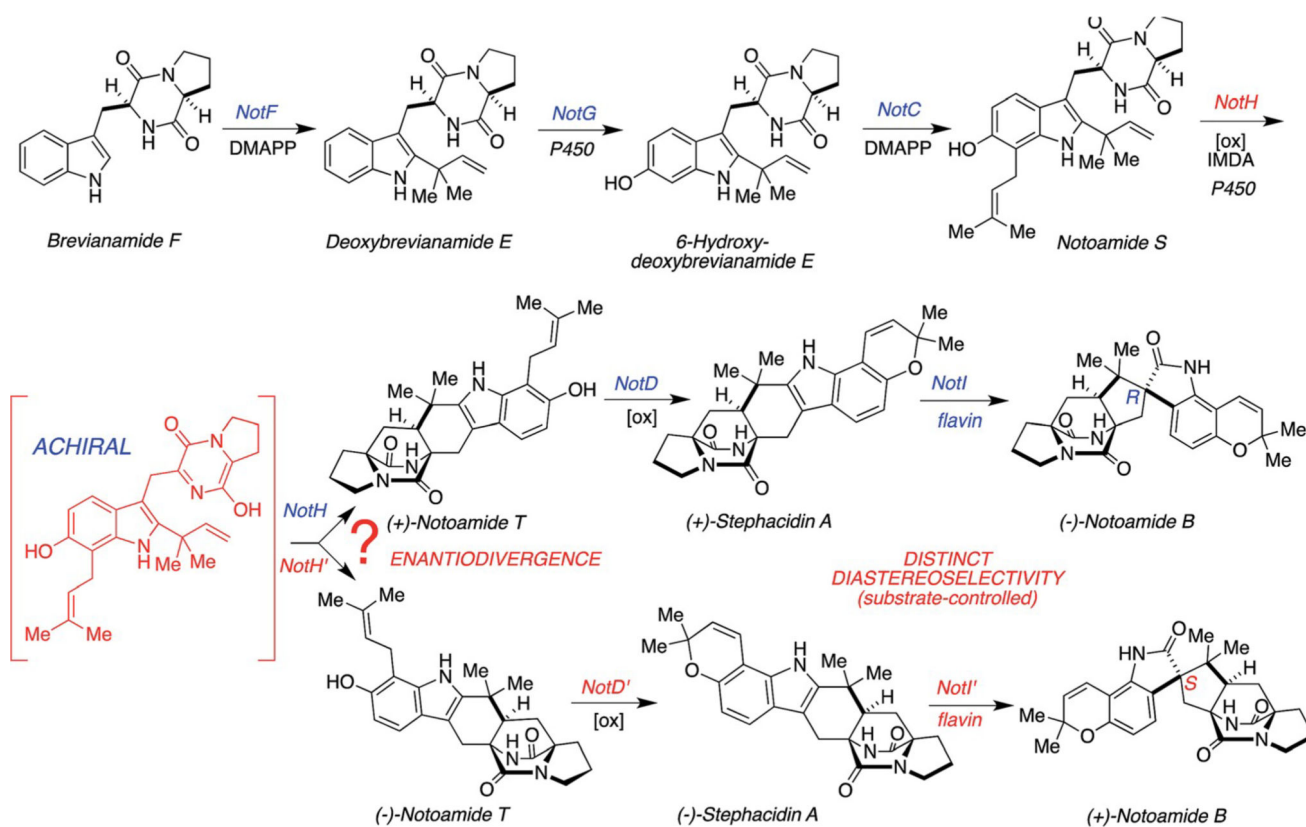
Possible biosynthetic pathways of an enantiomeric mixture of 6-*epi*-stephacidin A and (+)-versicolamide B in *A. amoenus* NRRL 35660.^{61,64}

**Scheme 11.**

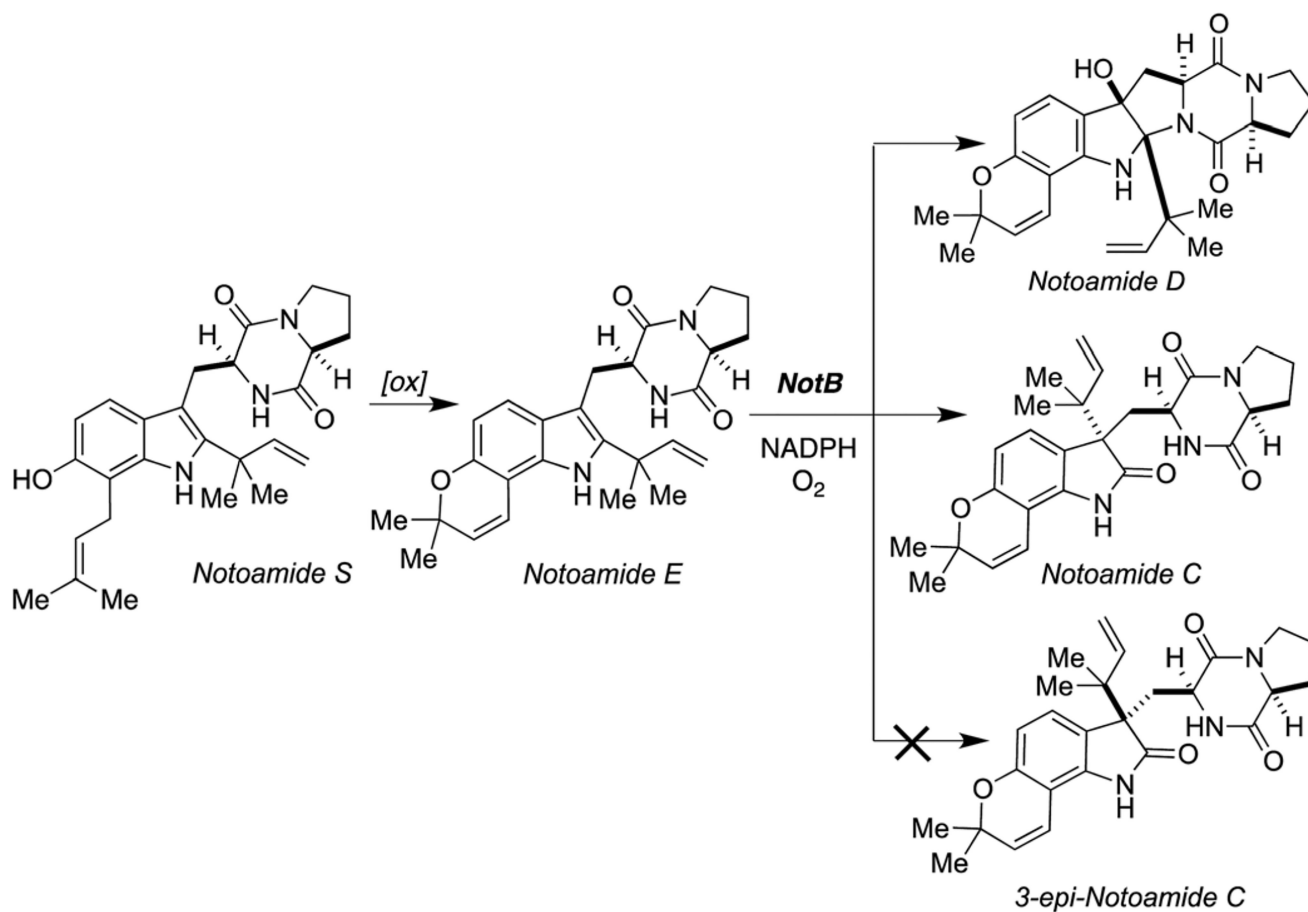
Possible biogenesis of taichunamides B and E and versicolamide B from 6-*epi*-stephacidin A.³⁰

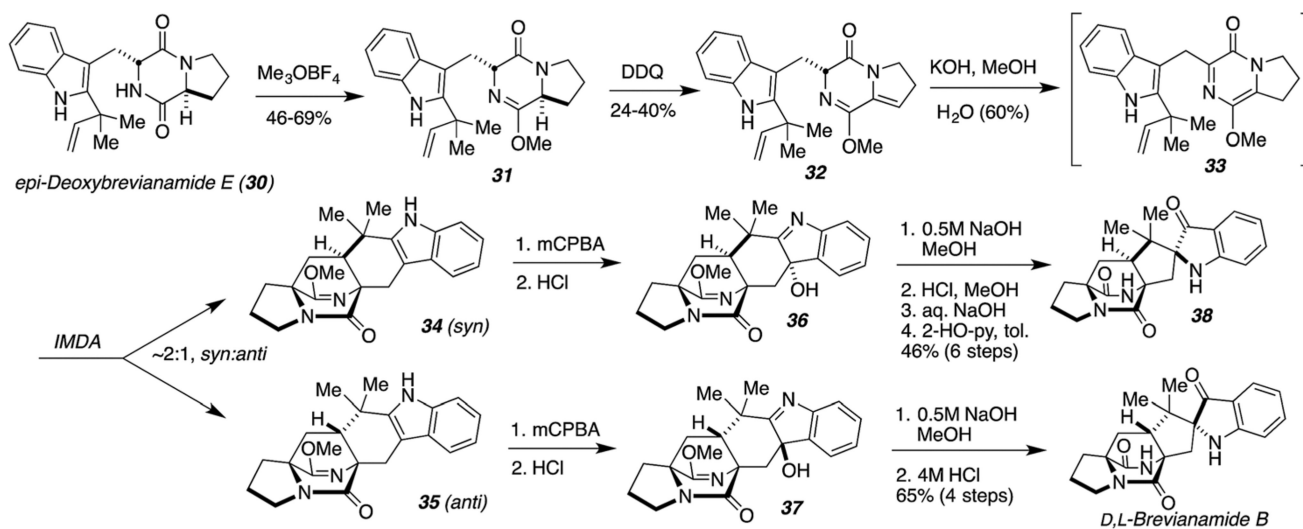
**Scheme 12.**

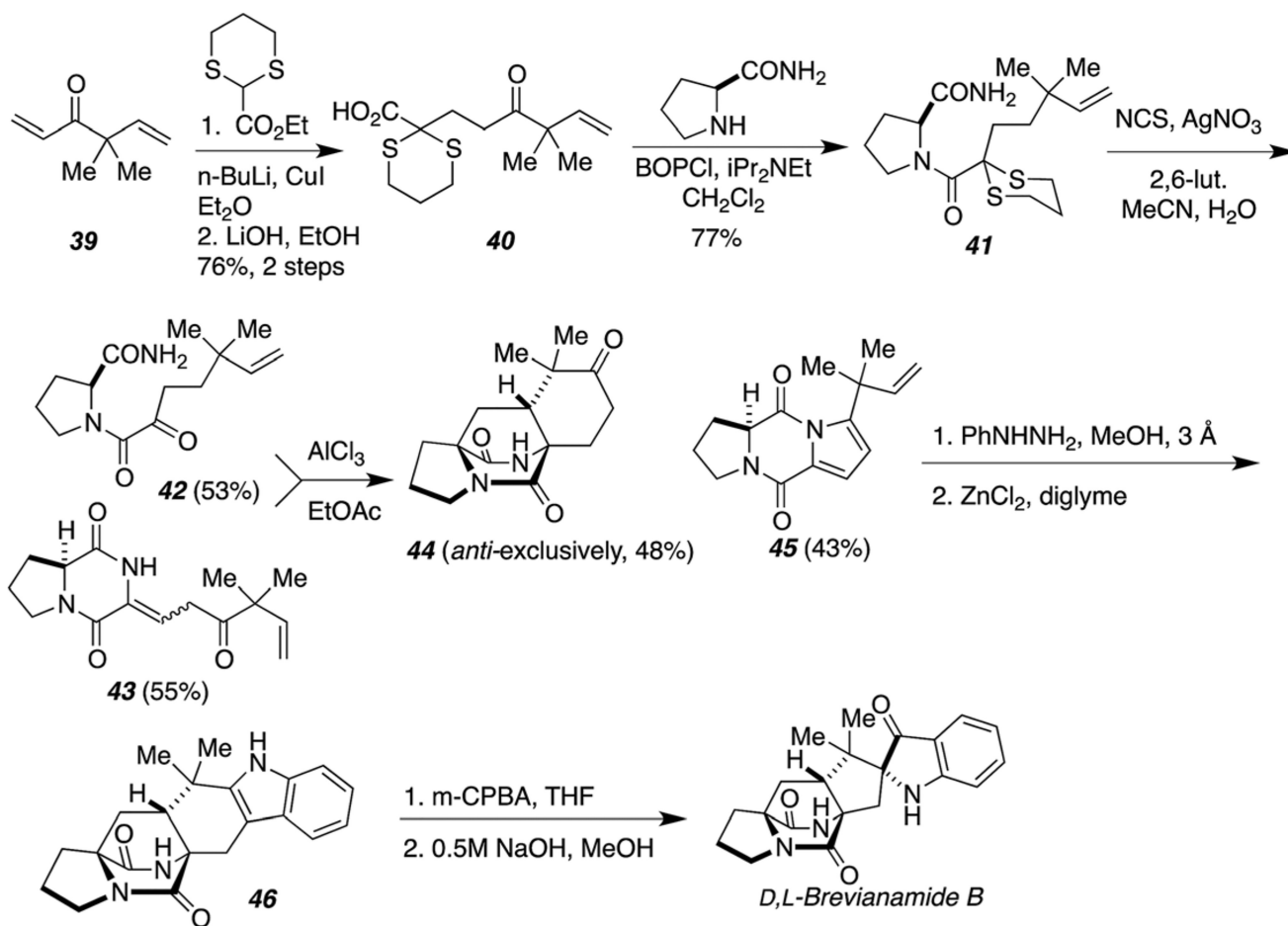
Proposed facial specificities of IMDA reactions for metabolites in *A. protuberus* MF297-2 (blue circles), *A. amoenus* NRRL 35660 (red triangles), and *A. taichungensis* IBT 19404 (green squares). Major and minor metabolites in each fungus are represented with large and small symbols, respectively.³⁰

**Scheme 13.**

Current understanding of (+)- and (-)-notoamide biogenesis in *Aspergillus* sp.⁶³

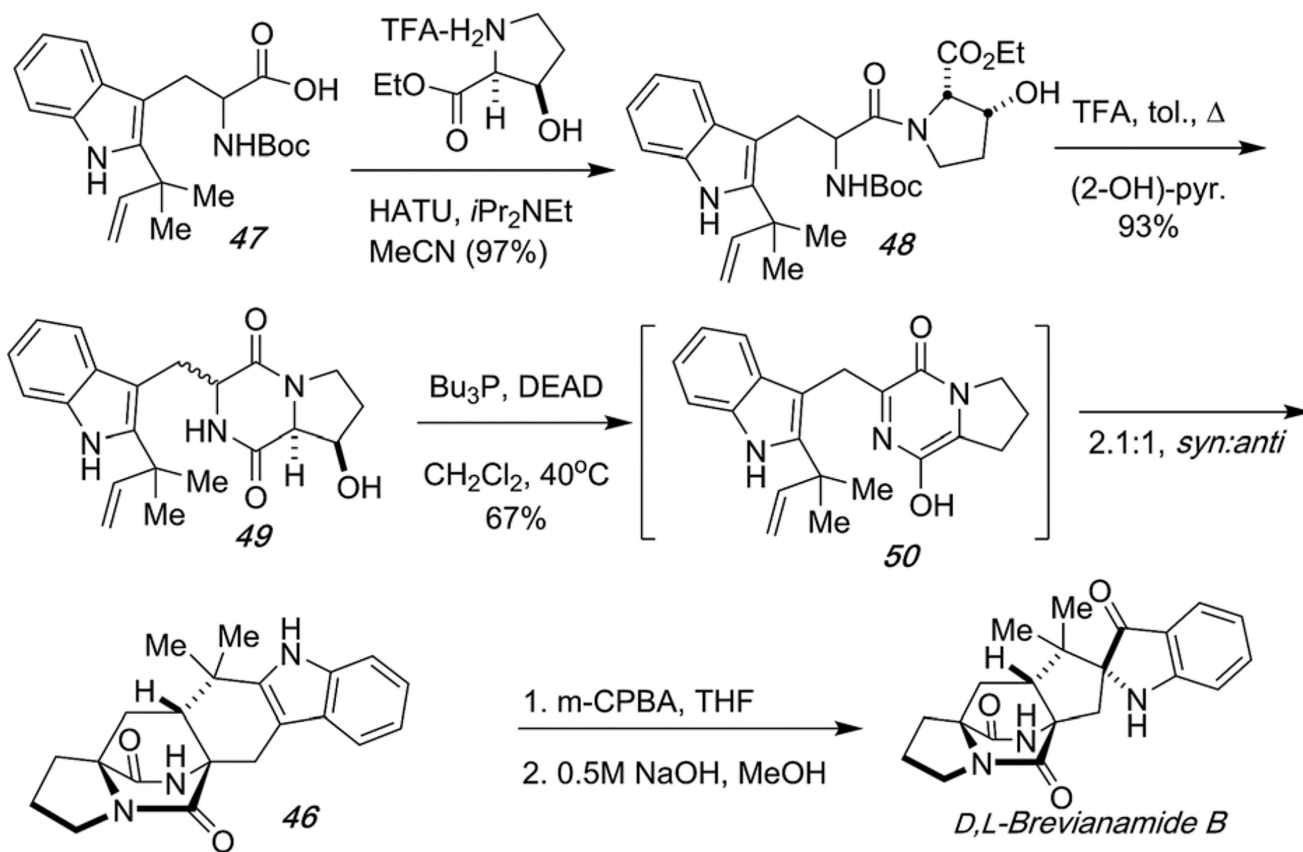
**Scheme 14.**Role of NotB in the conversion of notoamide E into notoamides C and D.⁷⁷

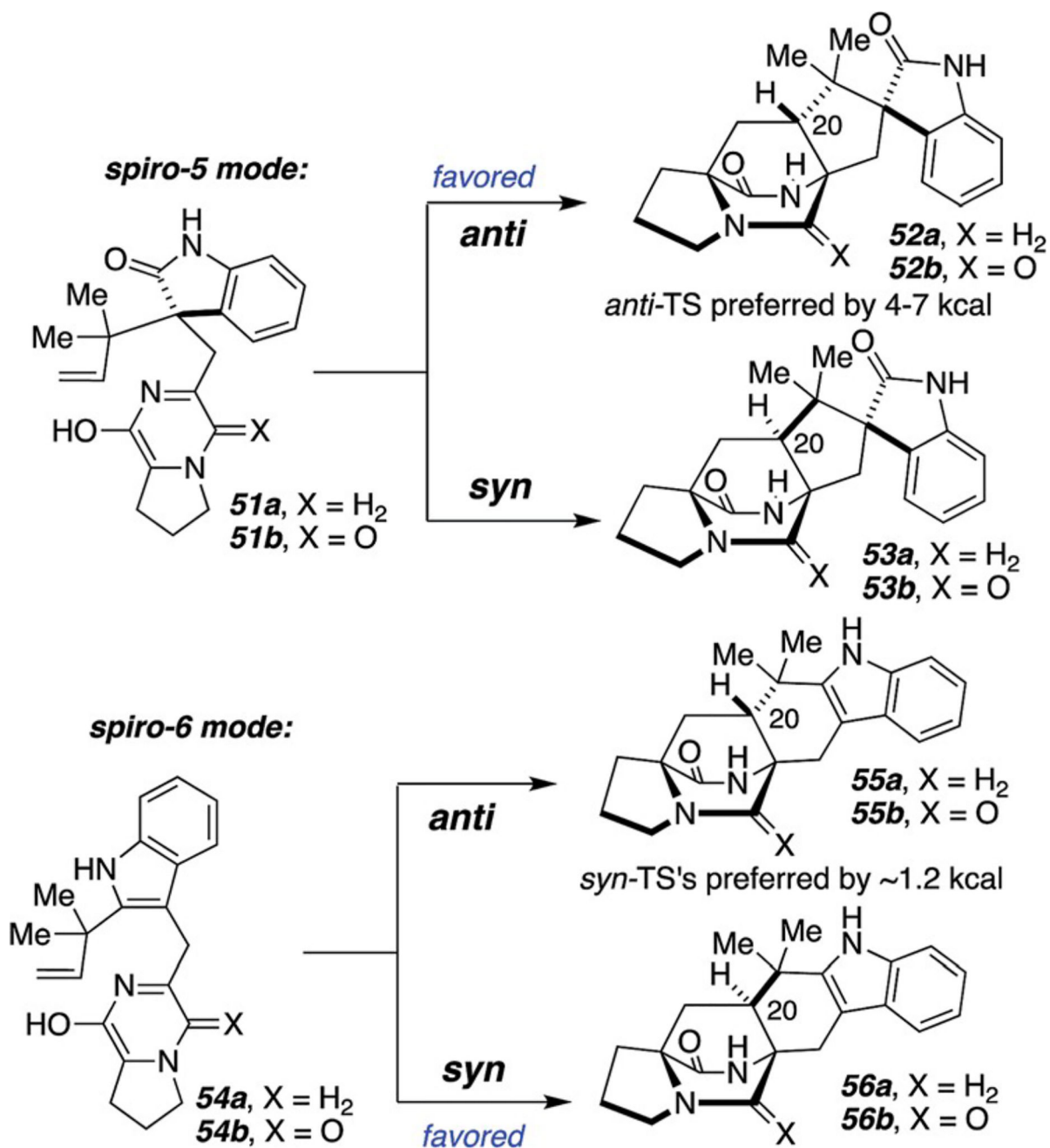
**Scheme 15.**First-generation biomimetic total synthesis of *D,L*-brevianamide B.⁸⁵



Scheme 16.

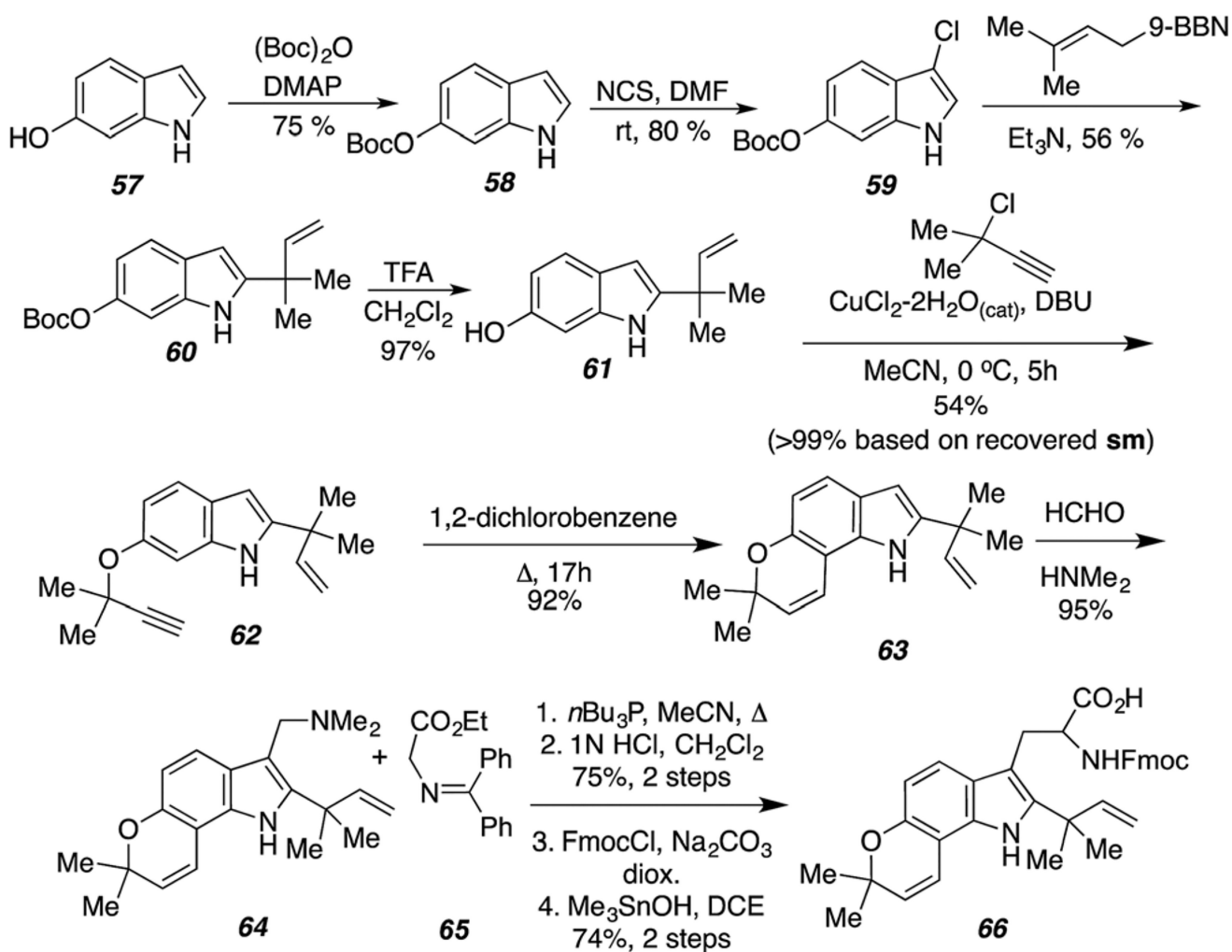
Second generation biomimetic total synthesis of *D,L*-brevianamide B.⁸⁶

**Scheme 17.**Third-generation biomimetic total synthesis of *D,L*-brevianamide B.^{60,85}



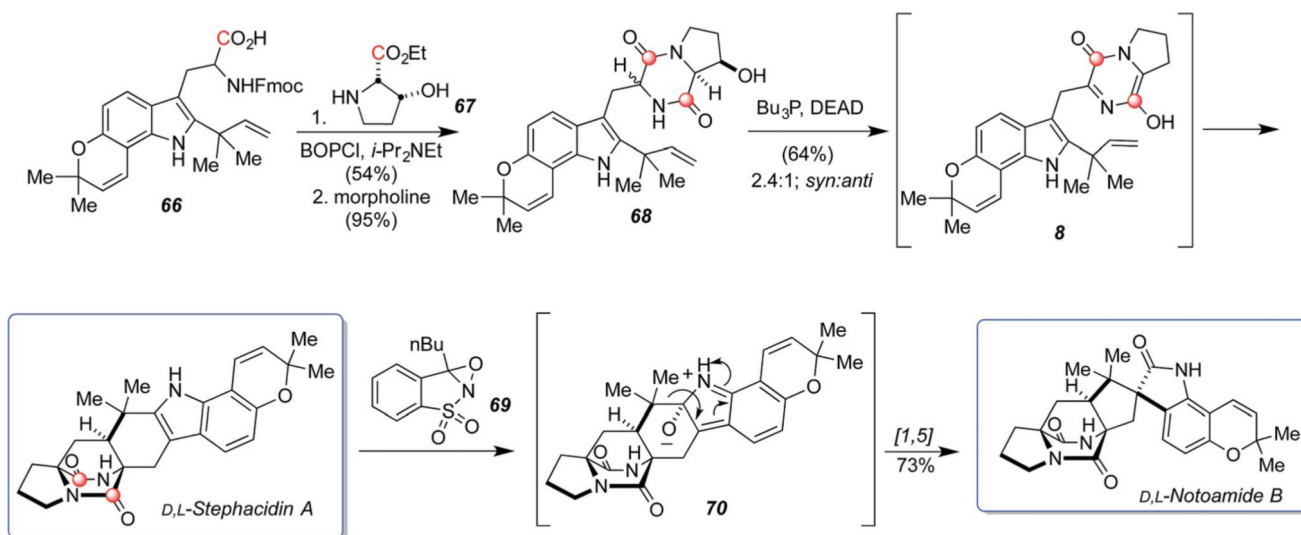
Scheme 18.

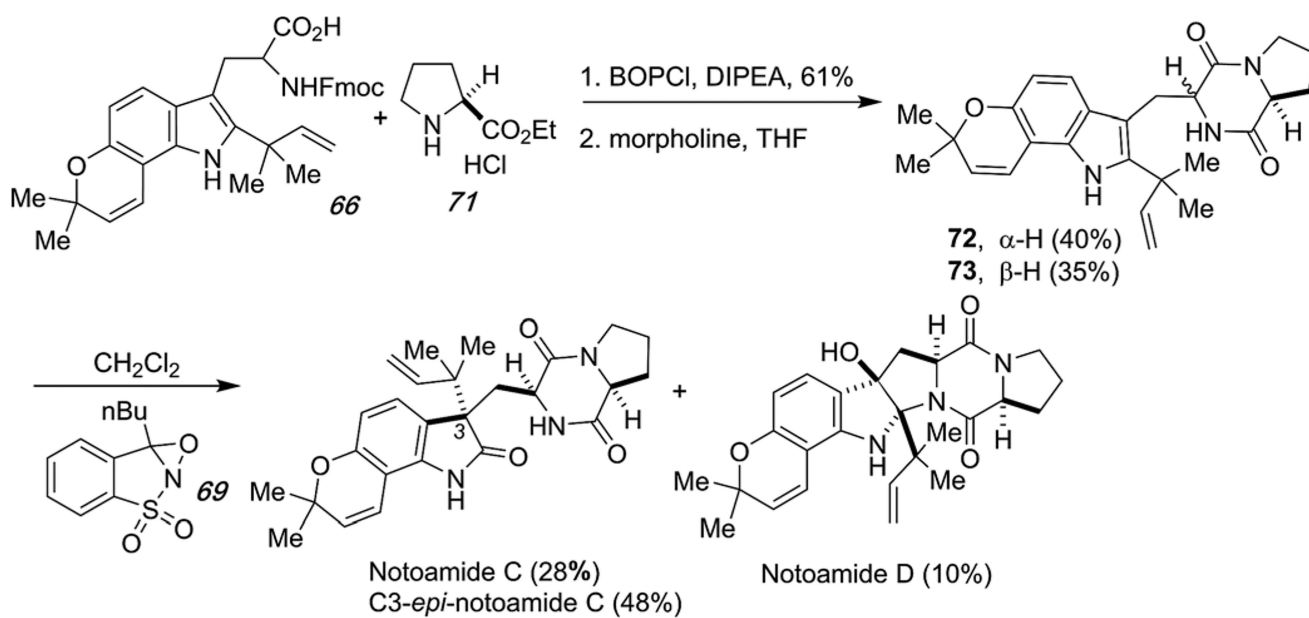
Theoretical calculations predicting transition state energies and predicted product diastereoselectivities at the B3LYP/6-31G* level.⁵⁴

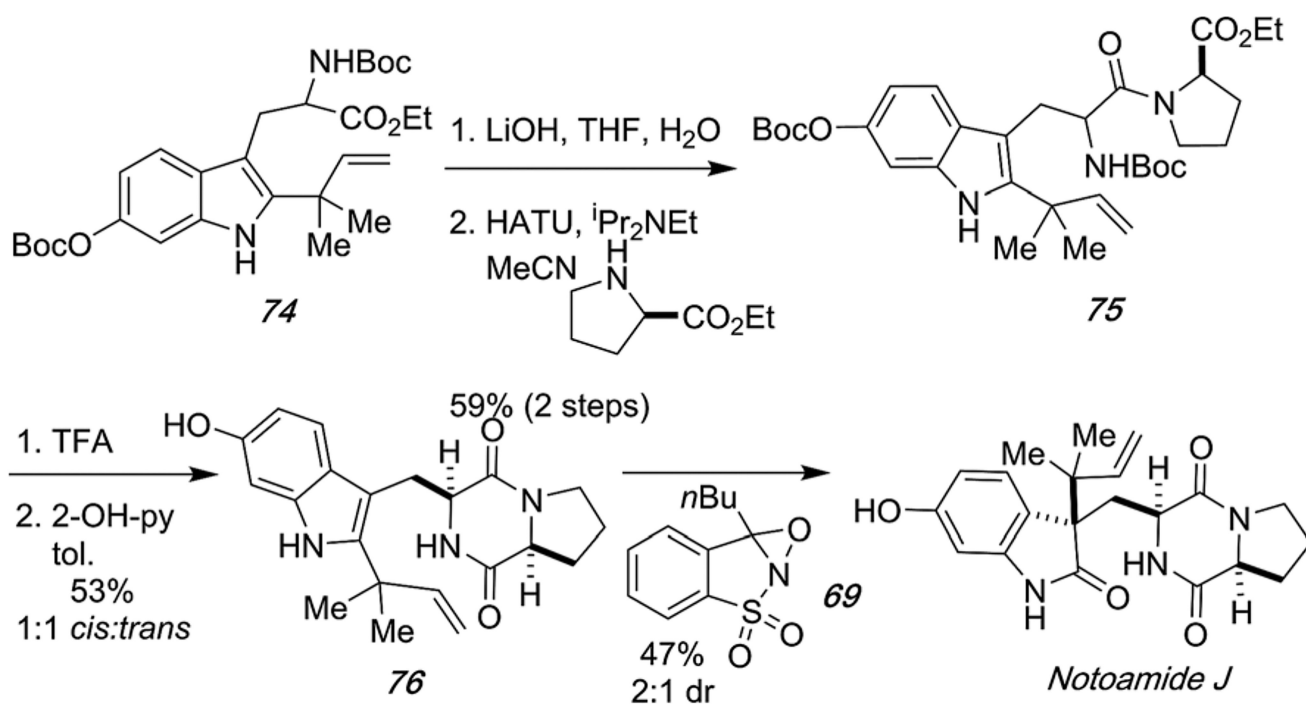


Scheme 19.

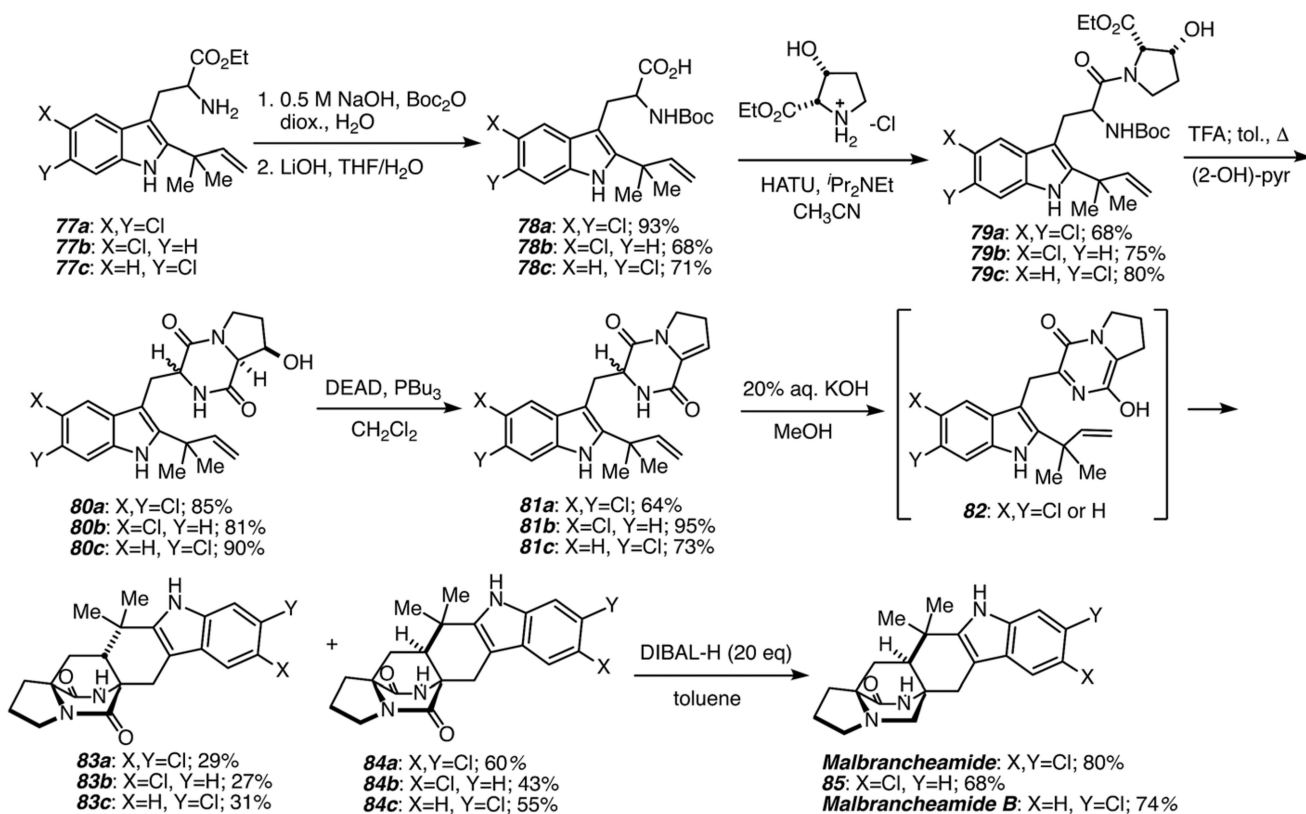
Synthesis of the reverse-prenylated tryptophan moiety.^{63,83,91–95}

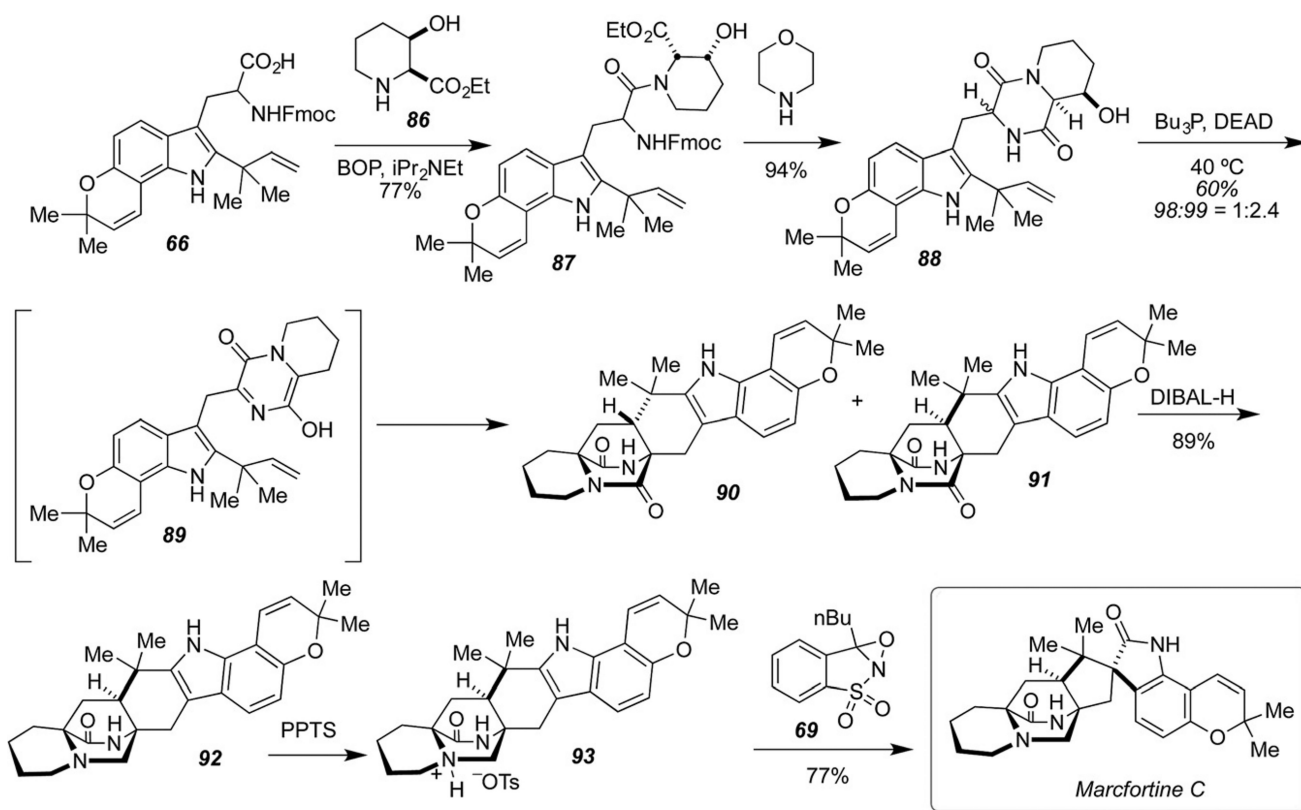
**Scheme 20.**Biomimetic total synthesis of stephacidin A and notoamide B.^{67,97}

**Scheme 21.**Biomimetic total syntheses of notoamide C, C3-*epi*-notoamide C and notoamide D.⁶⁶

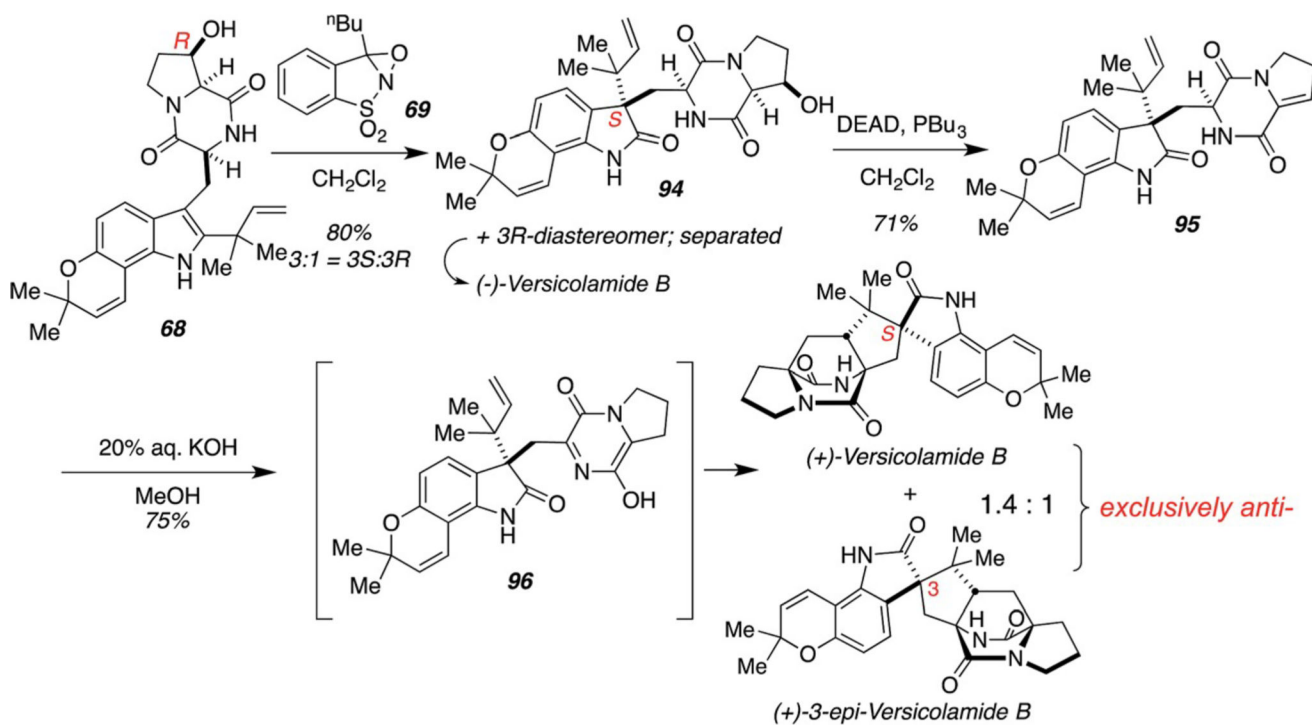


Scheme 22.
Biomimetic total syntheses of notoamide J.^{92,100}

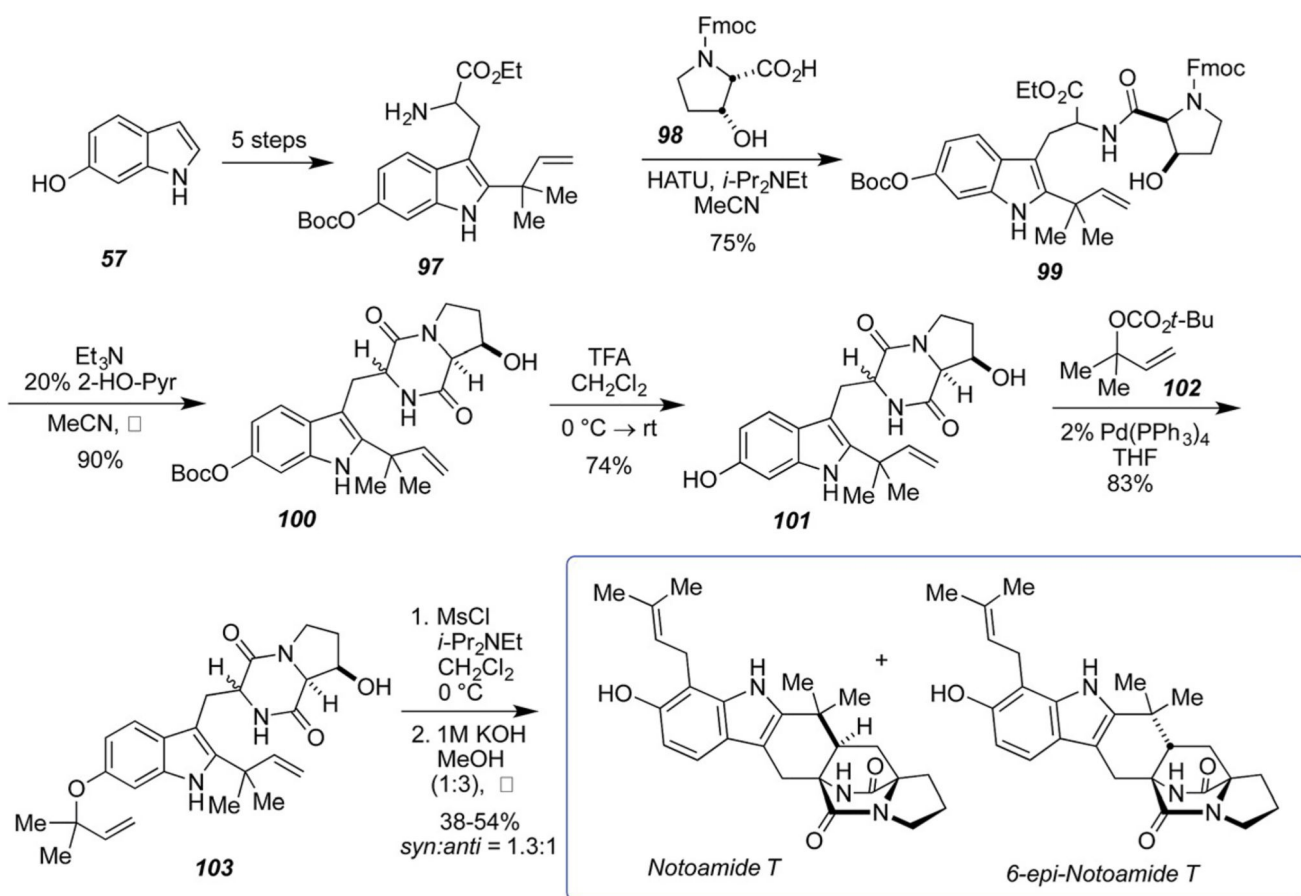
**Scheme 23.**Biomimetic total synthesis of the malbrancheamides.⁸⁸



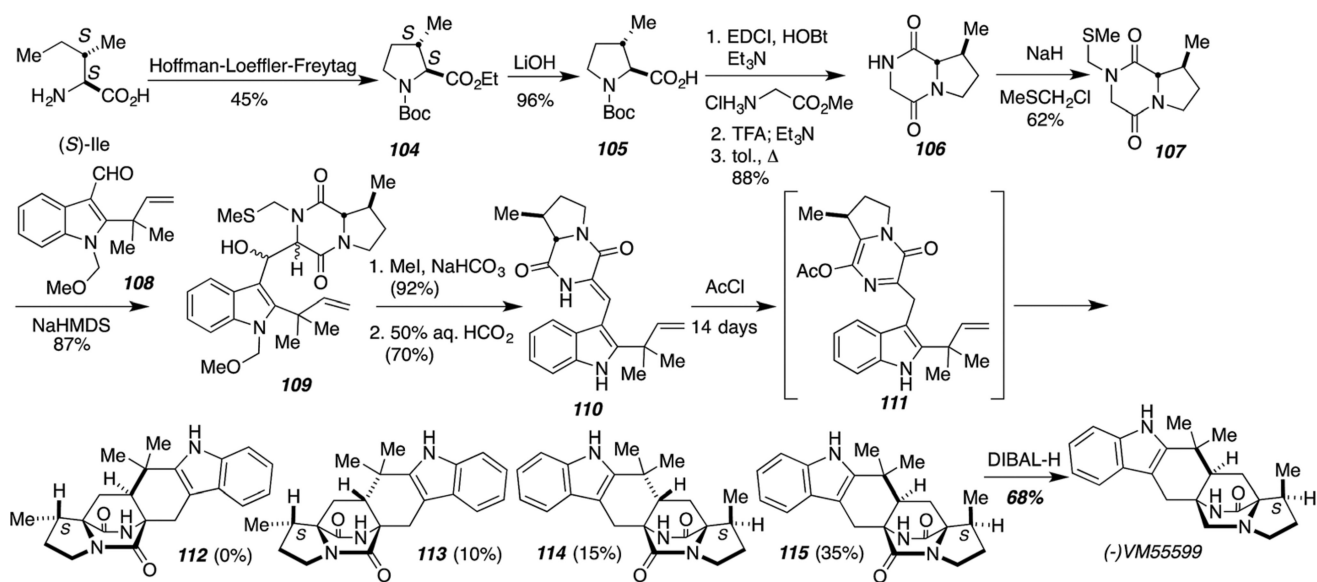
Scheme 24.
Biomimetic total synthesis of D,L -marcfortine C.⁹⁰

**Scheme 25.**

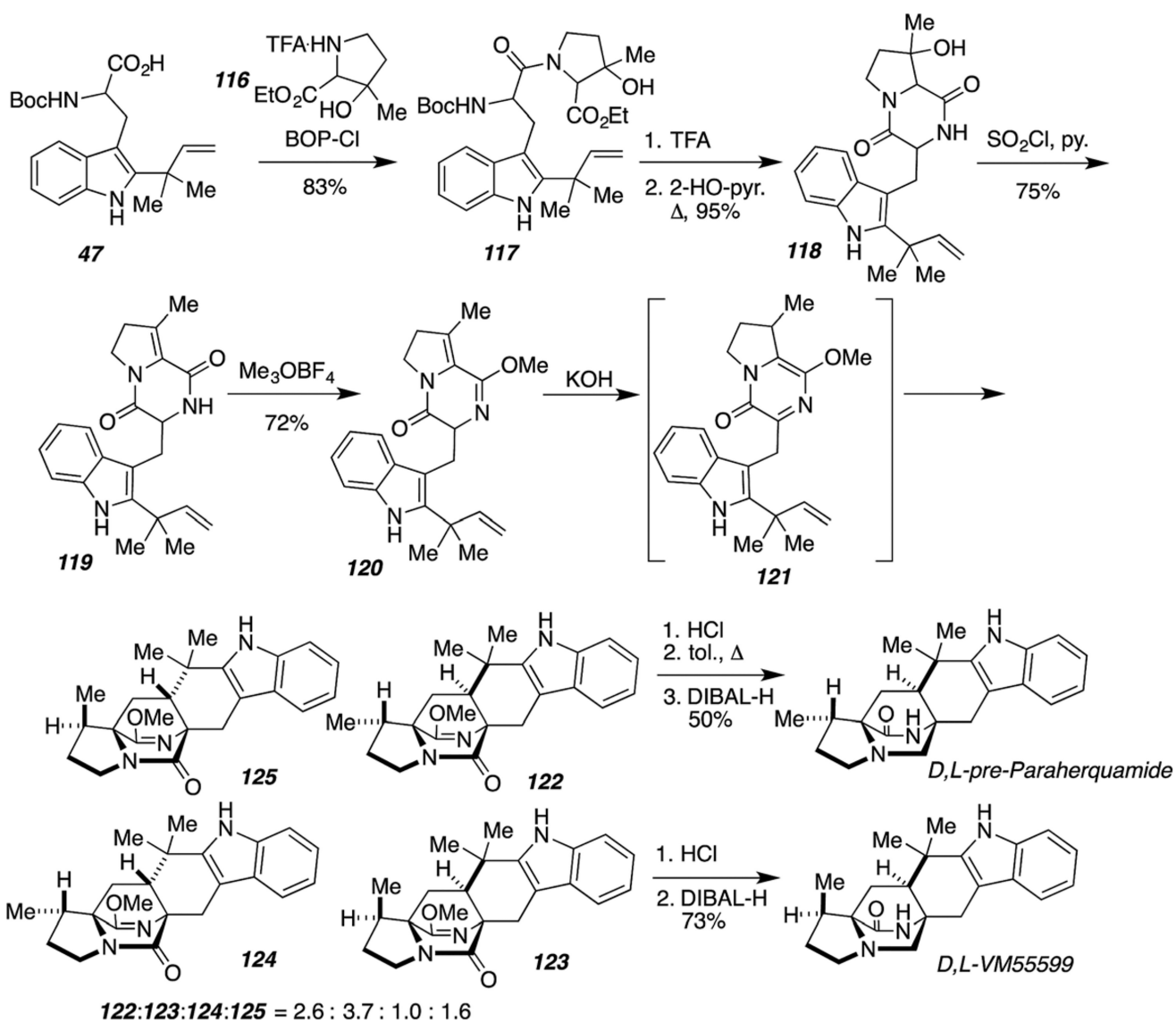
Biomimetic total synthesis of (+)-versicolamide B and (-)-versicolamide B.^{101,102}

**Scheme 26.**

Biomimetic total synthesis of D,L-notoamide T and D,L-6-*epi*-notoamide T.⁶³

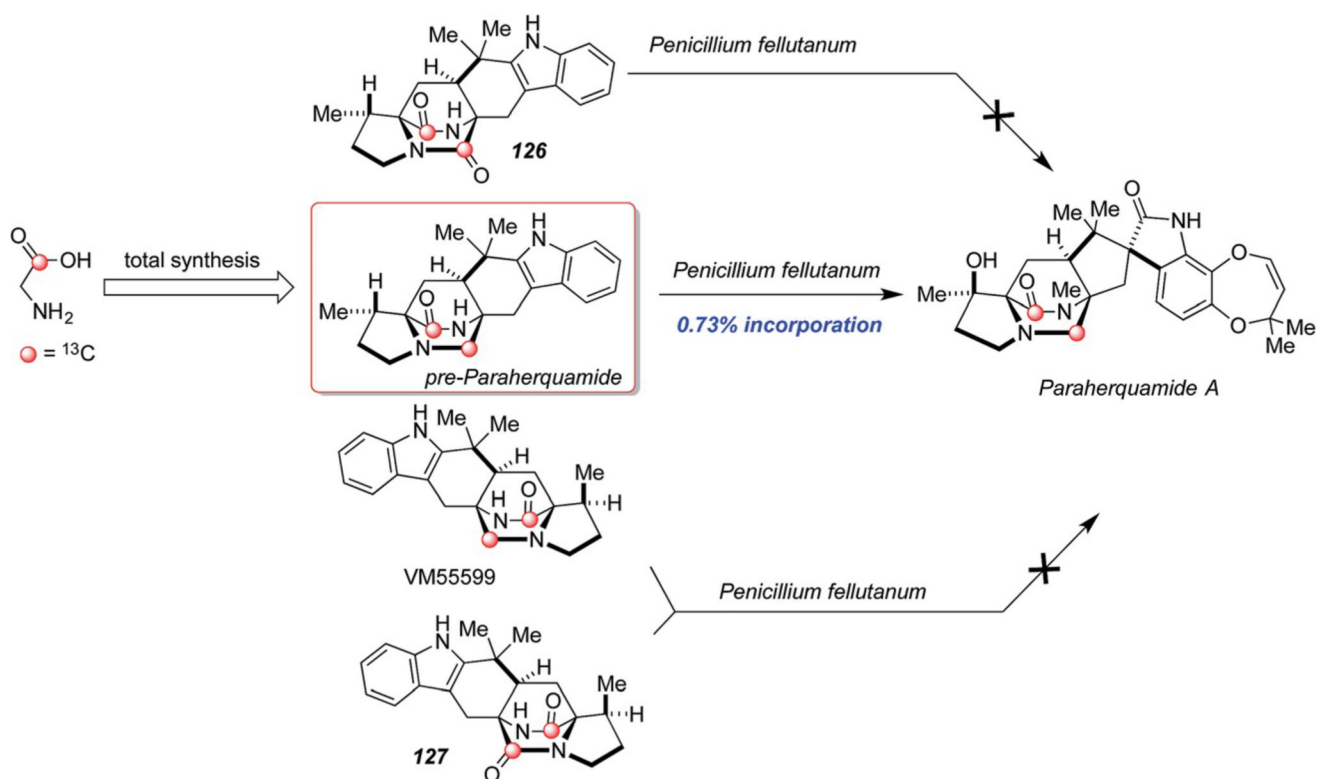


Scheme 27.
Biomimetic total synthesis of (-)-VM55599.⁵⁷

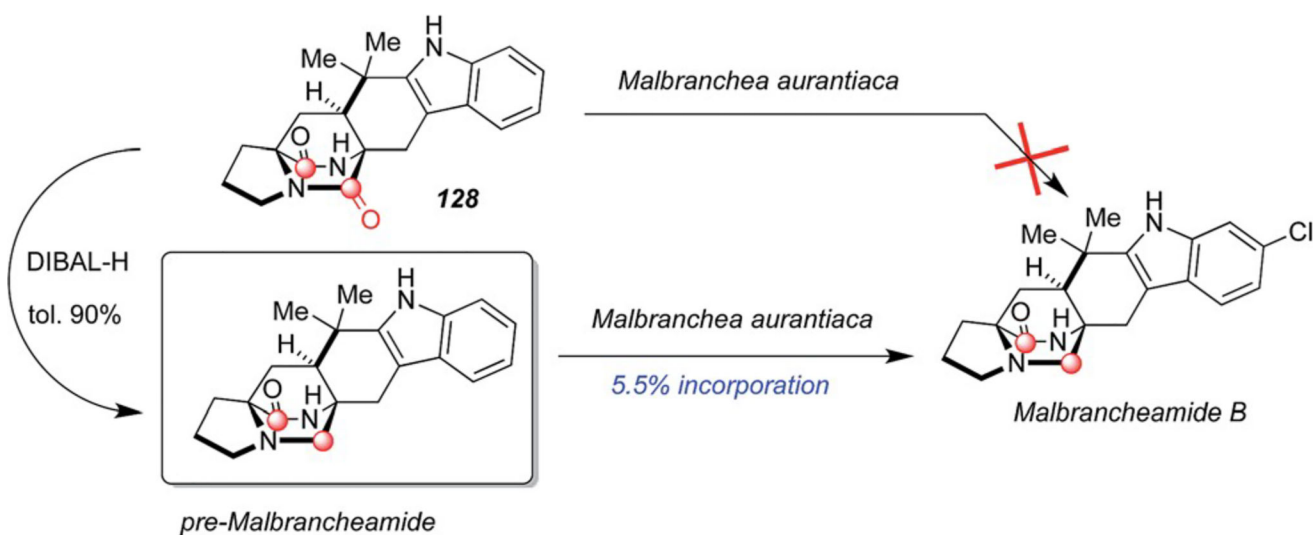


Scheme 28.

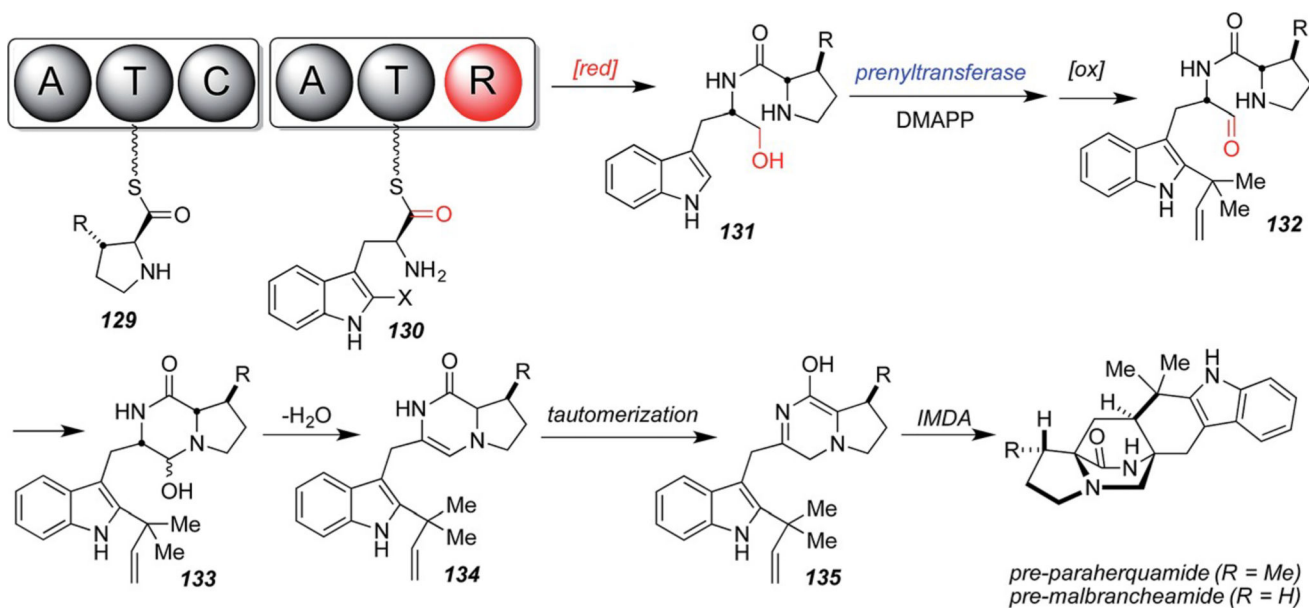
Biomimetic total synthesis of *D,L*-VM55599 and *D,L*-pre-paraherquamide.^{58,112}

**Scheme 29.**

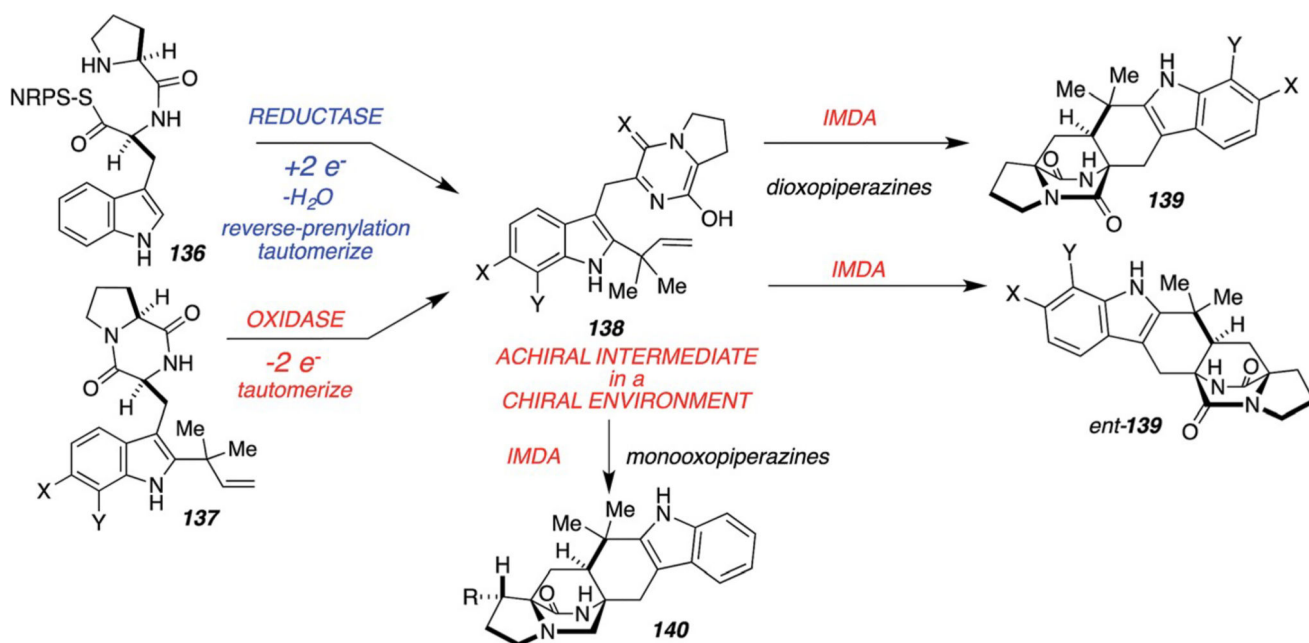
Results of precursor incorporation studies in *Penicillium fellutanum* with isotopically labeled dioxopiperazine and monooxopiperazine substrates.¹¹²

**Scheme 30.**

Results of precursor incorporation studies in *Malbranchea aurantiaca* with isotopically labeled dioxopiperazine and monooxopiperazine substrates.¹¹⁵

**Scheme 31.**

Molecular architecture of the monooxopiperazine NRPS module and a hypothetical biogenetic pathway to pre-parahequamide and pre-malbrancheamide.⁶²

**Scheme 32.**

Unified biogenetic hypothesis for prenylated indole alkaloids containing the bicyclo[2.2.2]diazaoctane nucleus.⁵³

Table 1

Select notoamide biosynthetic enzymes

Enzyme	bp/aa	Heterologous host	Function	Substrate
NotB	1563/456	<i>E. coli</i>	FAD binding domain protein	Notoamide E
NotB'	1344/455	-	FAD binding domain protein	Notoamide E
NotC	1350/427	<i>E. coli</i>	Prenyl transferase	6-Hydroxydeoxybrevianamide E
NotD	2025/621	<i>A. oryzae</i>	Oxidoreductase	(+)-Notoamide T
NotD'	2025/612	-	Oxidoreductase	(-)-Notoamide T
NotE	6723/2241	-	NRPS module	Pro + Trp
NotF	1431/453	<i>E. coli</i>	Reverse prenyl transferase	Brevianamide F
NotG	1901/544	-	Cytochrome P450	Deoxybrevianamide E
NotH	1836/502	-	Cytochrome P450	Notoamide S
NotH'	1836/499	-	Cytochrome P450	Notoamide S
NotI	1423/434	<i>E. coli</i>	FAD binding domain protein	(+)-Stephacidin A
NotI'	1423/433	<i>E. coli</i>	FAD binding domain protein	(-)-Stephacidin A